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COMPARATIVE EFFECTS OF CHLORTETRACYCLINE AND CORTISONE ON A LOCAL MONILIAL LESION IN THE MOUSE

BY

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The course of a local, closed monilial lesion in the thigh of the mouse is described. Treatment of animals suffering from this lesion with subcutaneous chlortetracycline in near-toxic doses leads to a persistent suppression of the lesion indistinguishable from a curative effect. Treatment with subcutaneous cortisone produces suppression of the lesion during treatment with subsequent "rebound."

Super-injection of the lesions with chlortetracycline produces a very large and persistent swelling. Treatment of these lesions with subcutaneous chlortetracycline in near-toxic doses produces suppression and "rebound" resembling that seen in lesions super-injected and treated with cortisone. Aspirin, chosen for its insolubility and acidity, and turpentine, chosen for its irritant effect, did not reproduce the effects of chlortetracycline or cortisone.

While it is possible that the suppressive effect of chlortetracycline and cortisone is in certain circumstances related, the local irritant effect of chlortetracycline plays an important part in the enhancement of these lesions.

Shortly after the introduction of penicillin, Geiger, Wenner, Axilrod and Durlacher (1946) described a case of generalized moniliasis following the administration of this antibiotic. Two years later, Weinstein (1947) drew attention to the spontaneous occurrence of new infections during the course of treatment with streptomycin or penicillin, and Zimmerman (1950) described further cases of generalized moniliasis following the use of these drugs. The possible importance of these findings was recognized and led some workers to explore the means by which these changes were brought about. Campbell and Saslaw (1949) claimed that the addition of streptomycin to the culture medium improved the growth of certain fungi, and Foley and Winter (1949) observed that the mortality rate of chick embryos and rabbits infected with *Candida albicans* was increased by treatment with penicillin.

Interest in these problems has been increased by reports of the frequent occurrence of moniliasis following treatment with chloramphenicol and chlortetracycline. The majority of those reporting these cases have supported the idea that overgrowth and increased invasiveness of monilia are dependent upon the suppression of normal bowel flora which accompanies the oral administration of these drugs (Tomaszewski, 1951; Woods, Manning and Patterson, 1951), and some *in vitro* results supporting this have been reported by Paine (1952). Some, however, believed that certain concentrations of the drug exert a direct stimulant effect on the growth of monilia (Moore, 1951) and it has been suggested that this effect is independent of antibiotic activity (Pappenfort and Schnall, 1951). Other workers have suggested that, either as a result of vitamin deficiency following the suppression of intestinal bacteria (Harris, 1950), or as a more direct effect of the drug (Seligmann, 1952, 1953), the defence mechanisms of the host are suppressed by chlortetracycline.

The possibility than an agent used for the treatment of infection may exert a depressant effect upon the defences of the host clearly deserves study. The work of Seligmann (1952, 1953) on the mortality of monilia-infected mice treated with chlortetracycline led him to postulate that chlortetracycline exerts an effect upon the response of the host analogous with that of cortisone. As a first step in the further study of this view we have compared the effect of cortisone and chlortetracycline on a measurable monilial lesion established in the mouse thigh. The object of this has been to see whether enhancement of the lesion

results from chlortetracycline treatment, and whether any changes which occur are comparable with those produced by cortisone.

Materials and Methods

Inoculation.—Two strains of *C. albicans* were used which had been recently isolated, one from a patient suffering from a urinary infection and the other from a patient suffering from vaginitis.

An overnight broth culture of the organism was flooded on plain agar plates which were then incubated at 37° for 48 hr. The growth was harvested with a spatula and suspended in normal saline. Direct counts of this suspension were made and a final inoculum containing 150 million organisms/ml. was prepared and used immediately. Purity of the inoculum was confirmed by culture.

Mice, in groups of 10, were inoculated intramuscularly in the left thigh as previously described (Selbie and O'Grady, 1954) with 0.1 ml. of this suspension using a 1 ml. syringe fitted with a 26 s.w.g. intradermal needle. Control groups of animals were similarly inoculated in the thigh with chlortetracycline 2 mg., 5 mg., or 15 mg.; cortisone acetate 1 mg.; acetylsalicylic acid 1 to 50 mg.; or turpentine 10 to 100 mg. Each drug was dissolved or suspended in 0.1 ml. normal saline. The medio-lateral diameters of the thighs were measured daily with sliding callipers and the average increase, compared with the normal thigh, calculated.

Subcutaneous Treatment.—The mice in different groups infected in this way received subcutaneous injections, in the flank at the time of infection and daily for 4 subsequent doses, of chlortetracycline 2 mg., 5 mg., or 15 mg.; cortisone acetate 1 mg.; acetylsalicylic acid 1 to 50 mg.; or turpentine 10 to 100 mg. Each dose of the drug was dissolved or suspended in 0.5 ml. of saline.

Intramuscular and Combined Treatment.—Further groups of animals were also infected in the thigh, but using 0.05 ml. of double strength inoculum (300×10^6 orgs./ml.), and an injection was immediately made into the infected thighs of chlortetracycline 2 mg. or 15 mg., cortisone 1 mg., aspirin 1 mg. or 50 mg., or turpentine 10 mg. Each drug was dissolved or suspended in 0.05 ml. saline and injected, where necessary, through a larger needle. These "super-injected" groups, therefore, received the same total volume of inoculum as those singly inoculated. Some groups of animals "super-injected" in this way were further treated subcutaneously with the same drugs at the time of inoculation and for 4 subsequent doses.

The various combinations of infection, intramuscular injection, super-injection, and treatment are summarized in Table I.

TABLE I

SCHEME OF TREATMENTS OF THE 17 GROUPS OF ANIMALS

With each substance, a number of dose levels were employed.

A. Intramuscular Injection	*B. Subcutaneous Treatment*
1. Monilia 2. Chlortetracycline 3. Cortisone 4. Aspirin 5. Turpentine	S.C. treatment with Monilia (given i.m.) { 6. Chlortetracycline; 7. Cortisone; 8. Aspirin; 9. Turpentine
C. Super-injection	*D. Combined Treatment*
Thigh "super-injected" with Monilia (given i.m.) { 10. Chlortetracycline; 11. Cortisone; 12. Aspirin; 13. Turpentine	Thigh "super-injected" with / Subcutaneous treatment with Same dose of Monilia (given i.m.) { 14. Chlortetracycline; 15. Cortisone; 16. Aspirin; 17. Turpentine

Results

Course of the Untreated Lesion

Infection of the thigh with *C. albicans* as described gave rise to a swelling which showed a peak size usually on the second day after inoculation followed by a small decline which was in turn succeeded by a second peak size usually about the 5th day. The size of the swelling then subsided to a plateau which might be maintained for a

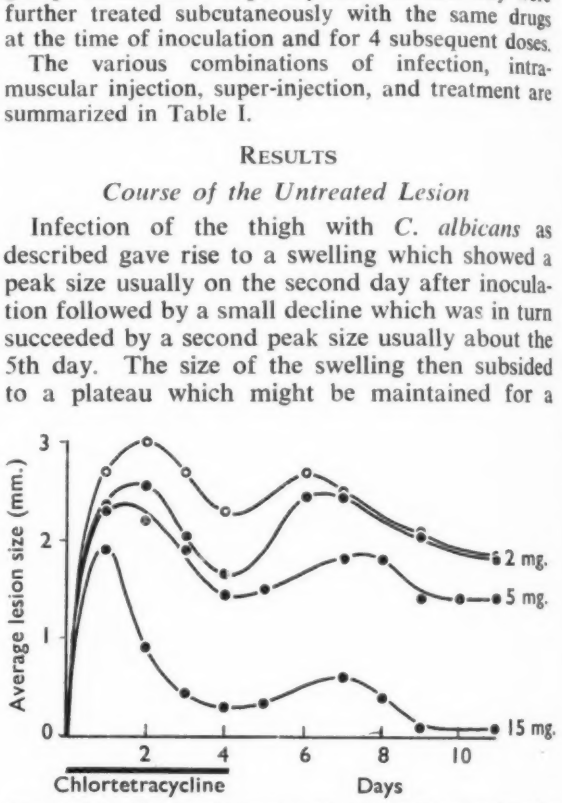

FIG. 1.—The effect of subcutaneous chlortetracycline at various dose levels on the course of the monilial lesion. O, Controls; ● Treated.

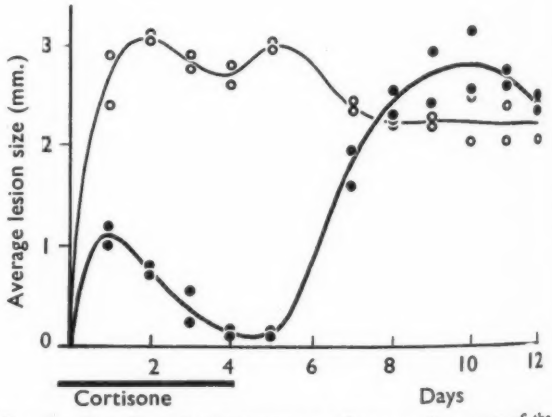

FIG. 2.—The effect of subcutaneous cortisone on the course of the monilial lesion. O, Controls; ●, Treated.

further week before resolution began (Figs. 1 and 2). The second peak might be greater than the first.

Microscopically, the lesion consisted of a granuloma within the connective tissue of the thigh. At the edge of the granuloma, proliferating monilial hyphae with some yeasts could be seen. The initial infiltration with polymorphs was soon succeeded by chronic inflammatory cells, histiocytes, macrophages and capillary loops. Pockets of pus containing numerous polymorphs and scanty monilia persisted, however, throughout the development of the lesions.

Effect of Treatment

Chlortetracycline

Subcutaneous Treatment.—The effect of subcutaneous chlortetracycline on the course of the lesion is shown in Fig. 1. Treatment at the 2 mg. dose level reduced the size of the lesion, but on cessation of treatment there was a return to the control size. Increasing the dose to 5 mg. reduced the size of the lesion still further and delayed its subsequent return to the control size. With toxic doses of chlortetracycline (15 mg.) swelling of the infected thigh disappeared by the third day and remained absent thereafter.

The largest dose of chlortetracycline given, 15 mg. for five doses, was very close to the cumulative LD50 for these mice and a number of deaths occurred amongst the treated animals. Subcutaneous chlortetracycline, particularly in these large doses, gave rise to marked inflammatory changes in the skin and subcutaneous tissue; infarcted areas were common and sloughing of the overlying skin sometimes occurred.

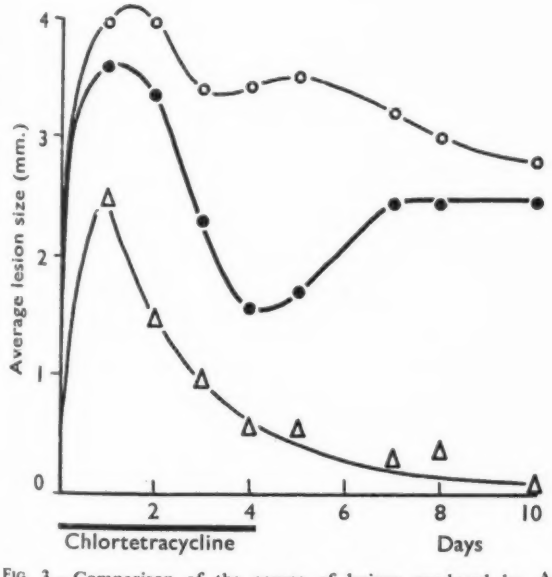

FIG. 3.—Comparison of the course of lesions produced by Δ, chlortetracycline (1 mg.); O, monilia plus chlortetracycline (1 mg.); and ●, monilia plus chlortetracycline (1 mg.) treated with subcutaneous chlortetracycline (1 mg.).

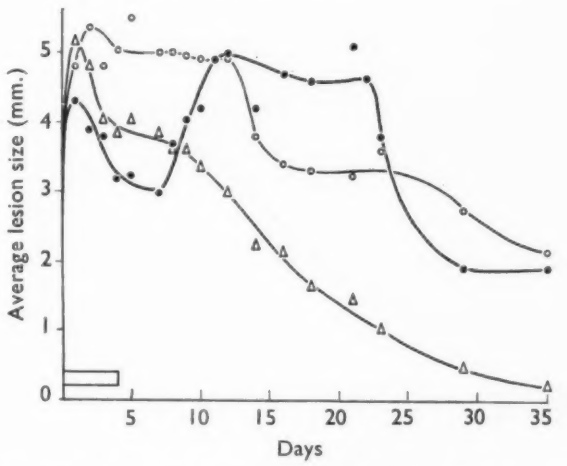

FIG. 4.—Comparison of the course of lesions produced by Δ, chlortetracycline (15 mg.); O, monilia plus chlortetracycline (15 mg.); and ●, monilia plus chlortetracycline (15 mg.) treated with subcutaneous chlortetracycline (15 mg.). The duration of treatment is shown by the hollow rectangle.

Intramuscular and Combined Treatment.—The intramuscular injection of chlortetracycline alone gave rise to a swelling maximal on the 1st day but which often showed a small secondary peak at 5 to 8 days. Super-injection of thighs already infected with monilia gave rise to very large and sustained swellings (Figs. 3 and 4). Subcutaneous chlortetracycline produced changes resembling those obtained in the animals only infected with monilia. When very large doses (15 mg.) of chlortetracycline were given (Fig. 4) the reduction in the lesion size persisted for 2 to 3 days after withdrawal of the drug, and was then followed first by a sharp rise to the control size and then by an increase above the control size, subsequently called " rebound."

Cortisone

Subcutaneous Treatment.—Five daily doses of 1 mg. cortisone acetate reduced the size of the lesions to zero, but following withdrawal of the drug there was a rapid " rebound " rise above the control size (Fig. 2). The difference between the means of the treated and untreated groups, calculated on day 10, is highly significant ($P<0.001$).

Intramuscular and Combined Treatment.—Intramuscular cortisone alone produced no recognizable swelling at 1 day; on the contrary, a

reduction in the size of the limb often occurred (Fig. 5). Injection of cortisone into limbs infected with monilia decreased the swelling for 2 days, but subsequently the swelling became greater than in the control animals. Subcutaneous treatment of doubly inoculated animals produced a reduction in size followed by "rebound" (Fig. 5). The difference between the means of the control and

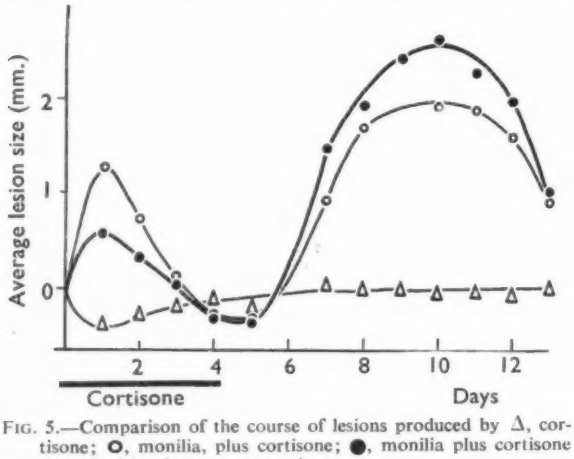

FIG. 5.—Comparison of the course of lesions produced by Δ, cortisone; O, monilia, plus cortisone; ●, monilia plus cortisone treated with subcutaneous cortisone.

treated groups calculated on day 10 is highly significant ($P<0.01$). This effect closely resembled that seen in animals treated with subcutaneous cortisone (Fig. 2).

Aspirin and Turpentine

Subcutaneous Treatment.—In doses up to and including the LD50 for these animals, these agents

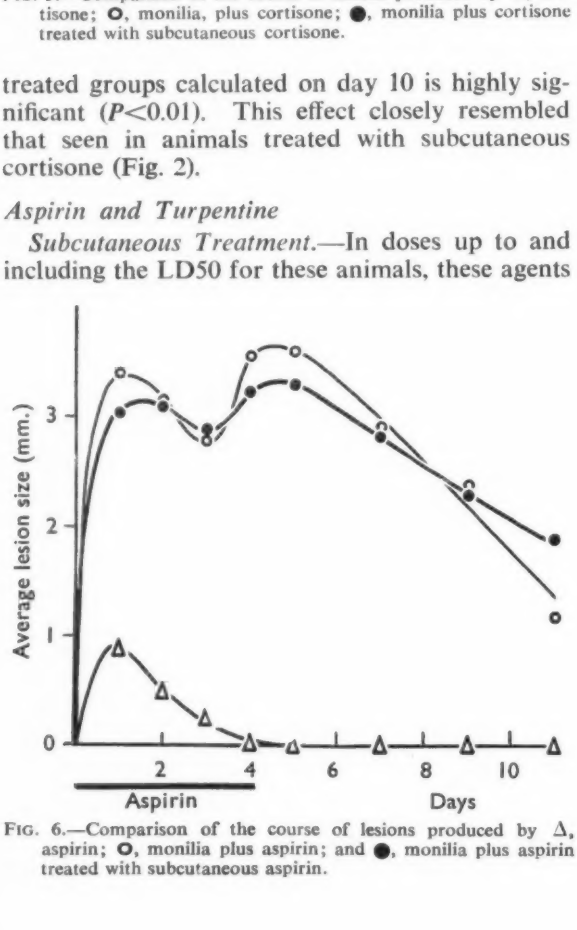

FIG. 6.—Comparison of the course of lesions produced by Δ, aspirin; O, monilia plus aspirin; and ●, monilia plus aspirin treated with subcutaneous aspirin.

produced only a small sustained reduction in the size of the lesions.

Aspirin

Intramuscular and Combined Treatment.—Very large doses of aspirin alone given into the thigh produced a small swelling only (Fig. 6). Super-injection of monilia-infected thighs gave rise to an insignificant increase in swelling, and in doubly inoculated animals the addition of subcutaneous treatment produced only minor changes (Fig. 6).

Turpentine

Intramuscular and Combined Treatment.—In contrast, the intramuscular injection of turpentine produced a very large swelling comparable with that resulting from the largest tolerated dose of chlortetracycline (Figs. 4 and 7). This swelling had its peak at the first day and subsequently declined logarithmically. Super-injection of infected thighs gave rise to a similar large swelling, but after the first 3 days, when the greater part of the swelling due to turpentine had subsided, the lesions followed the control course closely (Fig. 7). Treatment of such doubly inoculated animals with repeated subcutaneous turpentine was without effect on the course of the lesions apart from a small overall reduction in size (Fig. 7).

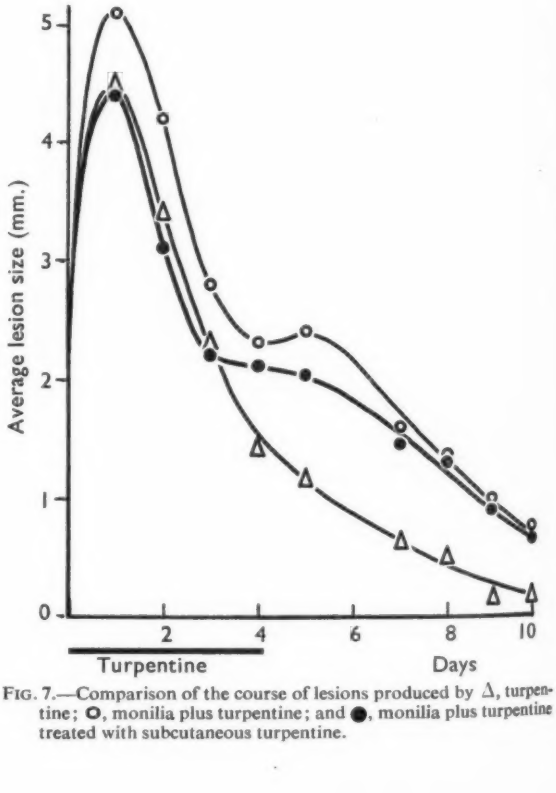

FIG. 7.—Comparison of the course of lesions produced by Δ, turpentine; O, monilia plus turpentine; and ●, monilia plus turpentine treated with subcutaneous turpentine.

Discussion

The possibility that chlortetracycline exerts a cortisone-like effect on monilial lesions has been examined using a monilial infection established at a site which is normally sterile. In this way, any changes which might be mediated by effects upon local normal flora have been excluded. The objects have been to determine whether the effects on this lesion of cortisone and of chlortetracycline are in any way similar, and to investigate to what extent the effects of chlortetracycline are the result of toxic or inflammatory processes.

Comparison of the effects of cortisone and chlortetracycline on the monilial infection reveals two similarities and two differences. The similarities are best seen in the animals receiving both intramuscular and subcutaneous injections (Figs. 3, 4, and 5). Both drugs produce a decrease in size which is followed by a rapid increase when treatment is stopped. In addition, if the chlortetracycline dose is high enough, this increase continues above the control value, as in those treated with cortisone (Figs. 4 and 5). This resembles closely the " rebound " increase or reappearance of activity which has been described in a number of conditions following suppression with cortisone (Ragan, 1950; Boland, 1950; Le Maistre, Tompsett, Muschenheim, Moore, and McDermott, 1951; Vollmer, 1951).

The first of the two differences is also shown by the groups receiving both intramuscular and subcutaneous injections. Intramuscular injection of cortisone alone produces a small reduction in the size of the thigh and in combination with monilia gives rise to swellings smaller than the controls (Fig. 5). Chlortetracycline, on the other hand, injected alone into the thigh produces a large swelling and in combination with monilia gives rise to a very large and persistent mass. The other difference occurs in the groups receiving the drugs subcutaneously only. The suppression and " rebound " resulting from cortisone typified in Fig. 2 cannot be reproduced by subcutaneous chlortetracycline in any dosage (Fig. 1). The reduction in lesion size which results from subcutaneous chlortetracycline becomes increasingly prolonged with increased dosage, but " rebound " does not occur even if chlortetracycline is given for two days only.

Both the important features of the response to cortisone can, therefore, be reproduced by chlortetracycline only in certain circumstances. There is, in addition, a local irritant response to chlortetracycline which is precisely the reverse of that to cortisone.

The possibility that this local irritant or toxic effect of chlortetracycline might be responsible for all the phenomena described led to a study of the effects of a number of unrelated substances chosen for their physical or biological properties. It is sufficient at present to say that the effects of cortisone and chlortetracycline have not been reproduced by any unrelated substance studied so far. Two examples of such substances have been chosen to illustrate some of the properties examined. Aspirin is a substance which corresponds fairly closely with chlortetracycline in acidity and solubility—physical properties of potential importance in the present connexion. Moreover, it has a therapeutic effect which resembles that of cortisone (Hailman, 1952). In the present experiments, it is clear that the action of aspirin does not resemble either that of chlortetracycline or that of cortisone.

Turpentine was chosen for its irritant properties, and sufficient was given to produce an irritant response in the thigh comparable with that of chlortetracycline. The failure of turpentine to reproduce the remainder of the effects of chlortetracycline makes it appear that chlortetracycline is not acting as a simple irritant. It must be observed, however, that neither aspirin nor turpentine gave rise to such persistent swellings as chlortetracycline, nor did either produce such severe tissue damage, though both were given to some groups in toxic doses.

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Brit. J. Pharmacol. (1958), **13**, 6.

SENSITIZATION OF THE HEART AND NICTITATING MEMBRANE OF THE CAT TO SYMPATHOMIMETIC AMINES BY ANTIHISTAMINE DRUGS

BY

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Sensitization to the cardio-accelerator action of adrenaline and noradrenaline by five antihistamines was examined on the acutely denervated heart of the cat. Antazoline (Antistin), chlorcyclizine and promethazine (Phenergan) increased the cardio-accelerator responses to both amines equally. Mepyramine (Anthisan) increased noradrenaline more than adrenaline action. Diphenhydramine (Benadryl) resembled cocaine in potentiating the responses to noradrenaline but not to adrenaline.

On the nictitating membrane mepyramine caused sensitization to the actions of adrenaline, noradrenaline and tyramine, an effect similar to that of chronic preganglionic denervation. Diphenhydramine enhanced the action of noradrenaline more than that of adrenaline and had little effect on tyramine action, giving a sensitization which bears a greater resemblance to the type caused by cocaine or chronic postganglionic denervation.

It is suggested that two distinct mechanisms are required to account for the phenomena of sensitization.

Enhancement of the action of adrenaline by antihistamine drugs has been described by Yonkman, Chess, Mathieson, and Hansen (1946) on the nictitating membrane of the cat, and by Sherrod, Loew, and Schloemer (1947) on the blood pressure of the dog. Antihistamines have also been shown to potentiate the inotropic action of adrenaline on the isolated toad heart (Kuriaki and Uchida, 1955). Lecomte and Fischer (1952) extended the observations on the nictitating membrane, finding enhancement of the action of other sympathomimetic amines bearing a phenolic hydroxyl group in the *meta* position. As the presence of a hydroxyl group in this position is characteristic of the amines whose action is augmented by cocaine, it was suggested that the potentiating action of the antihistamines was similar to that of cocaine.

Innes and Kosterlitz (1950) observed that, in cats with acutely denervated hearts, cocaine potentiated the cardio-accelerator action of noradrenaline but not of adrenaline. It was accordingly of interest to examine the effects of antihistamines upon the cardio-accelerator action of these amines.

It was shown by Innes and Kosterlitz (1954b) that ephedrine or chronic preganglionic denervation caused sensitization of the nictitating membrane to a wide range of sympathomimetic amines, while cocaine or chronic postganglionic denervation caused sensitization to some sympathomimetic amines but a reduced sensitivity to others. The present investigation therefore includes a study of the effects of two antihistamine drugs on the unsensitized, acutely denervated nictitating membrane and on membranes previously sensitized by chronic preganglionic or postganglionic denervation.

METHODS

Experimental.—For the analysis of the cardiac effects of the drugs, spinal cats were prepared by the technique described by Kosterlitz, Krayer and Matallana (1955). The heart was acutely denervated by cutting the vagi and removing the stellate ganglia and the upper part of the thoracic sympathetic chain. In experiments where the cardio-accelerator nerves were to be stimulated, the nerves were secured before removal of the stellate ganglia. In 3 cats the stellate ganglia were removed at a preliminary operation 16 to 28 days before the experiment.

The heart rate was counted from electrocardiographic recordings. A count of 30 sec. before injection of the sympathomimetic amine gave the basal heart rate. The injection was made over a 15 sec.

period and counting was continued for 90 sec. thereafter. In addition a continuous graphic recording was made by the heart rate recorder described by Innes and Kosterlitz (1954a). In this way prolongation of the action of a drug could be more readily observed.

In 3 experiments a Ritchie-BNI rectangular wave stimulator (Walter and Ritchie, 1945) was used to apply supramaximal stimuli to the cardio-accelerator nerves. Because the stimulus artefact prevented a satisfactory recording of the electrocardiogram, the heart rate increase during the 90 sec. period following stimulation was taken to be a sufficient measure of the response to stimulation of the nerves.

Care was taken to minimize the effects of temperature variation on the heart rate by ensuring that in each experiment the body temperature was kept constant within 0.5°.

In experiments where the effects of the antihistamine drugs were studied on the nictitating membrane of the cat, pentobarbitone sodium (50 mg./kg. intraperitoneally) was used as anaesthetic. The membrane was acutely denervated by preganglionic section of the cervical sympathetic trunk. Where the action on previously sensitized membranes was to be examined, the membranes were denervated preganglionically (decentralized) or postganglionically, by removal of the superior cervical ganglion (denervated) at a preliminary operation 14 to 16 days before the experiment.

In all animals the action of the adrenals was excluded, generally by removing the glands, but in a few experiments by tying the adrenal veins.

Drugs.—Suitable dilutions of adrenaline bitartrate, noradrenaline bitartrate and tyramine acid phosphate were made in Ringer-Locke solution containing ascorbic acid (0.4 mg./ml.). Injections of 0.25 to 1 ml. were made into the femoral vein, each injection taking 15 sec. The quantities refer to the free bases.

Solutions of antazoline HCl (Antistin), diphenhydramine HCl (Benadryl), chlorcyclizine HCl, mepyramine maleate (Anthisan) and promethazine HCl (Phenergan) were made in distilled water. The doses of these drugs and of cocaine HCl refer to the salts.

RESULTS

The Effect of Antihistamines on the Cardio-accelerator Action of Adrenaline and Noradrenaline.

Table I shows the potentiating effect of antihistamines on the cardio-accelerator action of adrenaline and noradrenaline on the acutely denervated heart.

TABLE I

POTENTIATION OF CARDIO-ACCELERATOR ACTION OF ADRENALINE AND NORADRENALINE BY ANTIHISTAMINES

The symbol + refers to an increase in the response to the amine. The asterisk refers to the results of Innes and Kosterlitz (1950, 1951).

Drug	Dose (mg./kg.)	No. of Experiments	Effects on Cardio-acceleration Caused by	
			Adrenaline	Noradrenaline
Diphenhydramine ..	5–10	3	0	+++
Mepyramine ..	1–5	5	+	++
Antazoline ..	1–3	3	+	+
Chlorcyclizine ..	5–10	3	+	+
Promethazine ..	2·5–20	4	+	+
Cocaine	2	*	0	+++

On the acutely denervated cat heart, noradrenaline normally causes little cardio-acceleration in comparison with like doses of adrenaline; after cocaine, noradrenaline action is increased to such an extent that it equals or exceeds the action of adrenaline (Innes and Kosterlitz, 1950, 1951). Of the antihistamines examined diphenhydramine alone caused a selective augmentation of noradrenaline cardio-acceleration as did cocaine (Fig. 1). Although mepyramine increased noradrenaline more than adrenaline action, a full cocaine-like effect was not obtainable, since the noradrenaline responses in no case became equal to those of adrenaline.

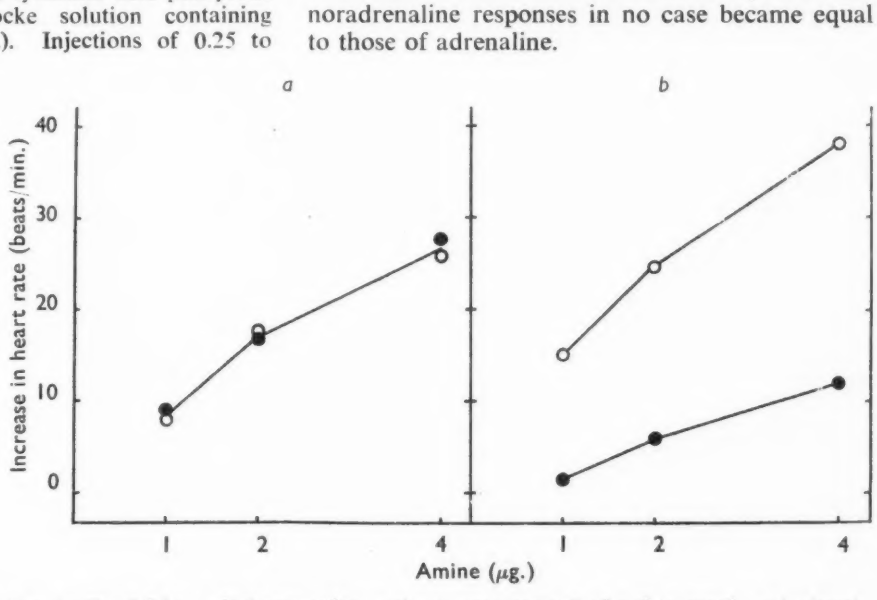

FIG. 1.—Cat, 2.6 kg., spinal preparation, vagi cut, acute removal of stellate ganglia and adrenals. Dose/response curves of increase in heart rate (beats/min.). ●——● control; ○——○ after diphenhydramine (10 mg./kg.). *a*, adrenaline; *b*, noradrenaline.

After the full effect of the antihistamines had been recorded, cocaine (2 mg./kg.) was injected in order to test whether a further increase in the noradrenaline responses was possible. Cocaine caused no further potentiation after diphenhydramine; with the remaining antihistamines, noradrenaline responses became equal to those of adrenaline only after the subsequent administration of cocaine.

The effect of mepyramine was examined in three cats on the heart sensitized by chronic denervation and in two cats on the heart sensitized by cocaine (2 mg./kg.). Both methods of sensitization had the usual effect of equalizing the responses to adrenaline and noradrenaline. Mepyramine caused no further increases in the responses. On the contrary, the addition of more than 2.5 mg./kg. depressed the responses.

The effect of mepyramine upon the responses to cardio-accelerator nerve stimulation was tested in three experiments. In each case mepyramine (5 mg./kg.) increased the responses to supramaximal stimulation; cardio-acceleration was increased from 37 to 48 beats/min. to 56 to 72 beats/min.

With the doses of amine used in this investigation the cardio-acceleration was usually of little more than 90 sec. duration. After administration of the antihistamines, the cardio-acceleration generally lasted not more than 90 to 120 sec. Thus these potentiating drugs did not markedly prolong the chronotropic action of the amines. Subsequent addition of cocaine (2 mg./kg.) extended the duration of cardio-acceleration to 4 to 5 min.

Kuriaki and Uchida (1955) found that diphenhydramine, perfused in a concentration of not less than 1×10^{-5}, caused a slowing of the heart rate in the isolated toad heart. Bradycardia was a constant feature in the action of all five antihistamines in the doses used in the present investigation. Antazoline, diphenhydramine and chlorcyclizine caused a slowing of 10 to 20 beats/min., while mepyramine and promethazine were more active, reducing the rate by 15 to 45 beats/min. The lower basal rate could not be regarded as an explanation for the increased activity of the sympathomimetic amines, for the enhancement was frequently much greater than the degree of slowing. For example, in one experiment diphenhydramine (10 mg./kg.) decreased the basal heart rate from 166 to 146 beats/min. while the cardio-acceleration due to 4 μg. of noradrenaline was increased from 12 to 58 beats/min. Moreover, mepyramine slowed the heart rate by 10 to 40 beats/min. in hearts sensitized by chronic denervation or by cocaine, while it caused no increase in the cardio-accelerator action of the amines.

The Effect of Antihistamines on the Action of Sympathomimetic Amines on the Nictitating Membrane.—In this investigation use was made of information derived from earlier studies of sensitization of the nictitating membrane (Lockett, 1950; Innes and Kosterlitz, 1952, 1954b). Two types of sensitization with different characteristics are known, one typified by the effect of chronic decentralization or of ephedrine, the other by the effect of chronic denervation or of cocaine. In the unsensitized, acutely decentralized or denervated nictitating membrane, adrenaline causes a greater contraction than does noradrenaline. Ephedrine or chronic decentralization brings about an unspecific increase in sensitivity to a wide range of sympathomimetic amines and does not affect the relationship between the sizes of the contractions due to adrenaline and noradrenaline. Cocaine or chronic denervation causes no further increase in adrenaline action but potentiates the action of many other amines bearing a phenolic hydroxyl group in the *meta* position. The action of noradrenaline is thereby increased to equal that of adrenaline. The effect of cocaine or chronic denervation on the action of amines lacking the hydroxyl group in the *meta* position is one of depression. By use of the three amines, adrenaline, noradrenaline and tyramine, the pattern of potentiation can be observed and an attempt made to classify the sensitizing effects of antihistamines into one or other of the types described above.

The effects of mepyramine and diphenhydramine were examined on the nictitating membrane, since these antihistamines gave the most pronounced results on the heart. It was especially important to study the effect of diphenhydramine in view of the likeness of its action to that of cocaine on the heart. The results are summarized in Table II.

TABLE II

POTENTIATION OF THE CONTRACTIONS OF THE NICTITATING MEMBRANE BY ANTIHISTAMINES

Comparison with effects of cocaine, ephedrine, chronic decentralization and chronic denervation. The symbol + refers to an increase in amplitude of contraction and the sign − indicates a decrease in amplitude of contraction. An asterisk refers to the results of Innes and Kosterlitz (1952, 1954b).

Antihistamine	Dose (mg./kg.)	No. of Experiments	Effect on Contractions of Nictitating Membrane Caused by		
			Adrenaline	Noradrenaline	Tyramine
Mepyramine ..	2·5–5	5	+	+	+
Diphenhydramine	5–10	4	+	++	0
Chronic decentralization		*	+	+	+
Ephedrine	0·03–0·1	*	+	+	+
Chronic denervation		*	+	+++	−
Cocaine	2	*	+	+++	−

The potentiation caused by mepyramine followed exactly the pattern of potentiation found in chronic decentralization ; the relationship between the sizes of the contractions was unaltered. This similarity to the effect of chronic decentralization was tested in two experiments with membranes sensitized by chronic decentralization. Mepyramine, in doses which ensured potentiation on the acutely decentralized membrane, caused no alteration of the responses of the chronically decentralized membrane.

Although diphenhydramine potentiated noradrenaline more than adrenaline contractions, a full cocaine-like effect was not attained. Adrenaline and noradrenaline responses were never equalized, while tyramine responses were little altered. Subsequent administration of cocaine showed that the full effect of equalizing adrenaline and noradrenaline action and depressing tyramine action could be produced in these cats. In two experiments with chronically decentralized membranes, diphenhydramine somewhat increased the sensitivity to noradrenaline, but without equalizing the response to adrenaline and noradrenaline. Tyramine responses were slightly reduced, but not to the same extent as after injection of cocaine. Diphenhydramine had no effect on the responses of chronically denervated membranes.

Discussion

In the sensitization of the heart which is exhibited by all five antihistamines examined, the antihistamine potency of the drugs appears to bear no relation to their potentiating activity. Mepyramine, promethazine and chlorcyclizine have been demonstrated by Marshall (1955) to exert greater antihistamine activity than diphenhydramine, while the present observations show diphenhydramine to be the most active in enhancing noradrenaline action.

Inhibition of amine oxidase by diphenhydramine has been demonstrated by Kuriaki and Uchida, who ascribe its sensitizing effect to this property. Amine-oxidase inhibition, however, is unlikely to play a role in the cocaine-like potentiation of sympathomimetic amine action, since Foster, Ing and Varagić (1955) found that no such potentiation on the nictitating membrane and blood pressure was caused by α-cocaine, a structural isomer of cocaine capable of inhibiting amine oxidase as effectively as cocaine.

It does not appear to be possible to link the ability to sensitize the heart with local anaesthetic activity, a property common to antihistamines and many other substances which potentiate sympathomimetic amine action. Diphenhydramine is a more effective local anaesthetic agent than mepyramine (Reuse, 1948), yet mepyramine potentiates adrenaline cardio-acceleration while diphenhydramine does not.

The possibility that two separate, and as yet unknown, mechanisms of sensitization are involved is suggested by the fact that diphenhydramine, like cocaine and chronic denervation, sensitizes the heart to noradrenaline but not to adrenaline action, while the other antihistamines sensitize the heart to the action of both amines.

The observations of the effect of diphenhydramine on the nictitating membrane agree with the work of Lecomte and Fischer (1952) showing that amines possessing a phenolic hydroxyl group in the *meta* position are potentiated by antihistamines. With mepyramine, however, the absence of the hydroxyl group in this position is not critical, as the action of tyramine was enhanced by this drug in each of five experiments in the present series. Thus mepyramine and diphenhydramine sensitization fall into different categories. Mepyramine causes an unspecific sensitization similar to the increased sensitivity found after ephedrine or chronic decentralization ; potentiation by diphenhydramine, on the other hand, more closely follows the sensitization caused by cocaine or chronic denervation, as it does in the heart.

In view of the existence of two distinct patterns of sensitization of the nictitating membrane it seems impossible to account for the phenomena of sensitization plausibly in terms of a single mechanism.

It appears therefore that two sensitizing mechanisms must be sought, one of which potentiates the actions of all sympathomimetic amines, 5-hydroxytryptamine (Innes and Kosterlitz, 1954b) and acetylcholine (Cannon and Rosenblueth, 1936), while the second further potentiates the actions of some sympathomimetic amines but suppresses the actions of others.

I am indebted for generous gifts of Antistin to Ciba Laboratories Ltd., of Benadryl to Parke, Davis and Co. Ltd., of chlorcyclizine to Burroughs Wellcome and Co., and of Phenergan to May and Baker Ltd.

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Brit. J. Pharmacol. (1958), **13**, 11.

ASSAY OF THE CAPILLARY ACTION OF FLAVANOID COMPOUNDS IN MICE

BY

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A method is presented for the assay of biologically active flavanoids by measurement of the reduction which these compounds cause in the sensitivity of the skin capillaries of mice to histamine. The assay is economical of time, space, and apparatus. Limits of error may be calculated from internal evidence. Time/effect relationships are also given for the capillary actions of rutin and hisperidin and for some of their derivatives.

The capillary actions of biologically active flavanoid compounds have hitherto been assayed in rabbits and in guinea-pigs. Three different criteria have been used for the measurement of the intensity of their action in these species. First, Bacharach, Coates, and Middleton (1942) used the effect of these compounds on the tensile strength of capillary walls. They measured the negative pressure required to produce petechial haemorrhages in guinea-pigs fed on a scorbutogenic diet supplemented with cod-liver oil and a known weight of ascorbic acid. Flavanoid compounds, administered orally, increased capillary strength. One month of treatment was needed before maximum discrimination between dose levels developed. The next criterion used was the antagonism by flavanoid compounds of the local increase in capillary permeability which results from irritation of the skin. Ambrose and de Eds (1947) made application of chloroform for 30 sec. to the depilated skin of rabbits, and Bohr, McIvor and Rinehart (1949) applied a brass plate at 58° for 30 sec. to the hairless skin of guinea-pigs to produce local irritation. Decrease in the resulting local change in permeability was measured as an increase in the time before coloration, due to leakage of dyes from the blood stream, appeared at the site of cutaneous irritation. Finally, Schiller (1951) made the potentiation caused by flavanoid compounds in the cutaneous vasoconstrictor actions of adrenaline and noradrenaline the basis of an assay in rabbits.

The assay method described here employs mice, and may be regarded as a modification of the rabbit method of Last and Loew (1947) for the assessment of antihistamine activity. The advantages of this mouse method are speed, economy of space, and simplicity. In addition, limits of error are calculable from the internal evidence of each assay.

METHODS

Male mice, weighing 20 to 35 g., varying by not more than 5 g. in each experiment, were used in groups of 6, 8, 9, or 10. Three different strains of white mice were used in the course of the work.

Flavanoid Compounds.—Rutin (Allen and Hanbury), Hisperidin, Hisperidin Methyl Chalcone, Quercitin, Naringin (a gift from Beecham Food Group), and Chalcone (Kodak) were dissolved in propylene glycol (B.D.H.). Volumes not exceeding 0.2 ml./mouse were injected by the intraperitoneal, and 0.075 ml. by the intravenous, routes.

The Dependence of Effect on Dose.—A separate group of mice was assigned to each dose level. A control group was assigned to each solvent volume. Each mouse in each group was treated as follows: (*a*) Test solution or solvent was injected at zero time. (*b*) After a fixed time interval (usually 20 to 30 min. for the intraperitoneal, and 15 to 20 min. for the intravenous, assay route) 0.15 ml. of pentobarbitone sodium solution containing 12 mg./ml. in aqueous 2% ethyl alcohol and 4% propylene glycol was injected intraperitoneally. (*c*) During 8 to 10 min. after (*b*), four well-separated intradermal injections of 1 μg. histamine acid phosphate in 0.02 ml. of 0.9% NaCl were made into the abdominal skin. (*d*) Ten minutes after (*b*), 0.1 ml. of a filtered 1% solution of Evans Blue in 0.9% NaCl was injected into the tail vein. (*e*) Fifteen minutes after (*d*), the mouse was rapidly killed. (*f*) Within two hours of death, the abdominal skin flap was resected and turned over so that the inner side lay uppermost. The depth of the blue colour was scored for each injection site by reference to a colour density chart. The total score for each

mouse was recorded. Colour density charts were made from smooth white paper, with 0.015% Evans Blue in water (w/v) and a paint brush. Successive washes of colour were applied to the paper, which was allowed to dry between each wash. Colour matching a single wash scored 1, a double wash scored 2, a treble wash scored 3, etc. The score range 3 to 9 was that suitable for use.

Variation of Effect with Time.—Four to six groups of 6 mice were injected with a fixed dose of test compound in a fixed volume of solvent; the same number of groups were injected with the solvent alone. Treatments (*b*) to (*f*) were applied to one test group and to one solvent group starting 1, 10, 20, 30, 40, and 60 min. after the initial injection.

Assay of the Capillary Action of Flavanoids.—Groups of not less than 9 mice are recommended. Two doses of standard (rutin) and two of test compound, which produced a submaximal, measurable, and comparable effect, were selected. The ratio of the high dose to the low dose was the same for standard and test compounds, and was normally 2.0. High doses were administered in 0.2 ml. and low doses in 0.1 ml. of propylene glycol intraperitoneally. Six groups of mice were required; one for each dose level of standard, of test, and of solvent. The effect of these doses on the resistance of the skin capillaries to the action of histamine was measured at the time of their maximum activity. This time had, in each case, been determined by previous experiment.

When no significant difference was found between either the mean score for each dose of test or of standard solution and the mean score for the corresponding solvent volume, the assay was discarded, and doses were readjusted before its repetition. This last has been necessary in two out of eighteen assays.

The assay procedure was modified when aqueous extracts containing naturally occurring flavanoid compounds were compared for their capillary action. Rutin in propylene glycol was again used as standard. Two additional groups of mice were injected with volumes of aqueous solvent corresponding to the volumes of test solution used. Differences between individual scores for mice treated with test and standard solutions and the mean score for mice correspondingly treated with solvent were expressed as % of those means. The values obtained were subjected to statistical analysis as described by Schild (1942). Corrections for coarse grouping were not applied.

Results

Rutin was selected for use in preliminary studies of the action of flavanoid compounds on the resistance of the skin capillaries of mice to the permeability changes induced by the intradermal injection of histamine, for the following reasons. Firstly, it was reasonably soluble in propylene glycol. Secondly, it was readily available in pure form. Thirdly, the capillary actions of rutin had been examined by all the methods formerly used for the assay of vitamin-P-like activity, and it was intended to use rutin as the reference compound for such assays in mice.

The Dependence of the Intensity of the Action of Rutin on the Dose Administered by Intraperitoneal Injection.—Measurement was made of the extent to which rutin antagonized the local increase in capillary permeability caused by the intradermal injection of histamine 45 min. after the intraperitoneal injection of this flavanoid. The combined results of three experiments are shown in Table I. The solvent, propylene glycol, itself caused some reduction in the responses of the vessels to histamine. This is made evident by comparison of the mean scores obtained after the intraperitoneal injection of the glycol and of 0.9% NaCl (w/v aqueous) into separate groups of mice. The effect of the propylene glycol alone was not, however, as great as when rutin was administered in it. The actions of rutin alone and of propylene glycol alone have been isolated in the last column of this Table. Both were graded in respect of dose, and that produced by rutin was linearly related to log. dose.

TABLE I

DOSE/EFFECT RELATIONSHIP FOR THE ACTION OF RUTIN (ADMINISTERED INTRAPERITONEALLY TO MICE IN PROPYLENE GLYCOL) ON CAPILLARY RESISTANCE TO HISTAMINE

Rutin/ Mouse mg.	Solvent ml.		Mean Scores/Mouse ±S.E. (No. of Mice)	
	Propylene Glycol	Saline	Recorded	Difference from Control Solvent
5	0·1	—	16·5±1·02 (18)	7·6 (propylene glycol)
10	0·2	—	10·4±0·86 (18)	10·0 ,, ,,
20	0·2	—	7·9±1·52 (18)	12·5 ,, ,,
—	0·1	—	24·1±1·46 (18)	3·5 (saline)
—	0·2	—	20·4±0·68 (18)	7·2 ,,
—	—	0·2	27·6±0·74 (18)	—

The use of propylene glycol as a solvent was made necessary by the limited solubility of many of the compounds under test. Its effects on the skin capillaries therefore received further study, and were finally attributed to the decrease in cutaneous blood flow and capillary pressure associated with its narcotic action. Increase in the depth of pentobarbitone anaesthesia similarly decreased the mean score/mouse in response to standard intradermal injections of histamine. The slopes of curves relating reduction in the histamine response to log. dose, given intraperitoneally, were very similar for these two narcotics. It was therefore theoretically possible to compute the reduction to be made in the dose of pentobarbitone for the use of a given volume of propylene glycol, such that the responses of the skin vessels should remain constant towards histamine.

In practice, however, the anaesthetic effects of propylene glycol were insufficient to allow of the necessary precision of high-speed intradermal injections. This finding made it necessary to limit the amount of propylene glycol used as solvent to 0.2 ml./mouse by intraperitoneal injection, and 0.075 ml./mouse by intravenous injection, and to include solvent controls in all quantitative experiments.

The dye, Evans Blue, was found to have capillary action. Intravenous injections of 0.1 ml. 1% Evans Blue made before intradermal injections of 1 μg. histamine acid phosphate in 0.02 ml. resulted in significantly less blue colour at the cutaneous injection sites than was found when the order of these injections was reversed (Table II). This made

TABLE II

THE EFFECT OF INTRADERMAL HISTAMINE ON SKIN PERMEABILITY IN THE PRESENCE OF EVANS BLUE

Time of Injection of Dye	Mean Score/Mouse±S.E. (No. Mice)
2 min. before histamine	14·3±2·86 (8)
1 ,, after ,,	26·8±0·76 (8)
2 ,, ,, ,,	27·2±1·32 (8)

it necessary to control the times of injection of both the histamine and the dye strictly during quantitative work. First, intradermal injections of histamine were made in a time interval not exceeding 40 sec. Then the Evans Blue was injected intravenously within the interval of 1 to 1.5 min. after the last histamine injection.

Post-mortem examination of mice which had received intraperitoneal injections of rather insoluble flavanoids dissolved in propylene glycol 1 hr. previously, for example hisperidin, frequently revealed the presence of finely divided flavanoid precipitated in the peritoneal cavity. Since the intraperitoneal injections of suspensions of talc in 0.9% NaCl were without effect on the responses of the cutaneous vessels to intradermal injections of histamine, it was concluded that the most accurate possible method of expressing the activity of flavanoid compounds administered by the intraperitoneal route would result from measurement both of the intensity and duration of their action. In practice, time/effect relationships have been determined at a single dose level, and potency has been measured at the time of maximum activity. This procedure was adopted for economy of test material, mice, and time.

Finally, since this rapid assay method was likely to find its use in assisting the isolation of naturally occurring substances with vitamin-P-like action from vegetable extracts, the possibility that well-recognized groups of organic compounds might interfere with the responses of skin capillaries to histamine was briefly reviewed. The intraperitoneal, but not the intravenous, injection of glucose, fructose, and cane sugar caused the gradual onset of a period of decreased sensitivity of the skin vessels to histamine. This change was demonstrable in 15 min., reached maximum at 30 to 40 min., and had decreased but was still significant at 60 min. The intensity of change appeared to be more closely related to the total amount of sugar injected than to the tonicity of the solution used. The magnitude of this change is illustrated by the results of a typical experiment in Table III.

TABLE III

THE EFFECT OF GLUCOSE AND FRUCTOSE SOLUTIONS, INJECTED IN A VOLUME OF 0·4 ML. INTRAPERITONEALLY, 30 MIN. BEFORE TESTING SENSITIVITY OF SKIN CAPILLARIES TO HISTAMINE

Solution Injected Intraperitoneally	Mean Score/Mouse
Saline	25·8±3·6 (6)
Glucose 6% in water	22·3±2·4 (6)
,, 6% in saline	22·7±2·1 (6)
Fructose 6% ,,	22·5±3·1 (6)
Glucose 12% in water	16·7±1·9 (6)
,, 12% in saline	16·2±2·3 (6)
,, 6% and fructose 6% in water	16·3±2·4 (6)
Saline	25·4±1·9 (6)

The Dependence of the Action of Flavanoid Compounds on the Weight Administered Intravenously.—The intravenous injection of 10 mg. rutin or of 5 mg. hisperidin in 0.15 ml. propylene glycol into the tail veins of mice was immediately followed by cyanosis. Microscopic examination of the lungs and mesentery showed that many capillaries became blocked by precipitated flavanoid within 2 min. of the injections. This observation made worthless estimates of the capillary actions of flavanoids characterized by a low solubility in water and administered intravenously.

Aqueous extracts containing water soluble flavanoid glycosides from various fruits caused reductions in the sensitivity of skin vessels to histamine which did not appear for 10 to 20 min. after their intravenous injection. The intensity of the capillary action of these extracts was, when fully developed, related linearly to log. dose.

The Relationship between Intensity of Action of Flavanoid Compounds on the Skin Capillaries and Time after Administration.—The delayed onset of the capillary actions of water soluble flavanoid compounds after intravenous injection indicated that changes brought about in the chemical structure of these compounds by metabolic processes conferred or greatly enhanced their capillary actions. Time/effect curves of flavanoid compounds of known structure were therefore studied

for the purpose of discovering, within the limitations of the meagre series available, the effect of change in chemical structure on this latent period.

The structural unit common to all flavanoid compounds is 2-phenyl benzopyrone. Most of those found in fruit are glycosides in which the sugars, usually glucose and rhamnose, are attached to the aglycone in positions 7 and 3. Three commonly occurring types of flavanoid compounds differ in their structure at position 3; these are the flavones, the flavanols, and the flavanones. The flavones were represented by the glycoside rutin and its aglycone quercitin in the series studied. The glycosides hisperidin and naringin represented the flavanones. None of these glycosides had sufficient solubility in water to permit measurement of the latent period of their capillary action by intravenous injection. They were given dissolved in propylene glycol by the intraperitoneal route. Two chalcones, hisperidin methyl chalcone derived from a flavanone, and chalcone derived from the flavone rutin, were also examined. These compounds and also quercitin were sufficiently soluble to be studied both by intravenous and by intraperitoneal administration.

Time/effect relationships for all these compounds are shown in Table IV. Reduction in the sensitivity of skin capillaries to histamine appears in this Table as a reduction in the mean score/mouse for the blue colour which leaked from the capillaries at the sites of intradermal injections of histamine. The time course of the action of the flavone glycoside rutin resembled very closely that of the flavanone glycoside hisperidin. Capillary action did not appear for 20 min. and developed to a maximum in 40 min. after the intraperitoneal injection of these compounds. The time course for the action of the aglycone quercitin was indistinguishable from that of the parent glycoside rutin administered by intraperitoneal injection, whether the quercitin was given by the same or the intravenous route. It was therefore concluded that sugar molecules attached to the aglycone neither hindered absorption from the intraperitoneal cavity nor delayed activation of the aglycone by metabolic processes. By contrast, reduction in the sensitivity of skin capillaries to histamine appeared within 10 min. of either the intraperitoneal injection or the intravenous injection of chalcones derived from both hisperidin and rutin. These chalcones, especially the highly purified methyl derivative of hisperidin, had intense but short-lived action by intravenous injection and only small activity when given into the peritoneal cavity. This indicated their breakdown to inactive metabolites in the liver. Finally the action of hisperidin methyl chalcone was shown to have reached maximum intensity within 3 min. of its intravenous injection. The evidence obtained from these time/effect studies was therefore compatible with the hypothesis that the glycosides and aglycones owe their capillary action to chalcones formed from them in the animal body.

Flavanoid Compounds Failed to Antagonize the Action of Histamine on Smooth Muscle.—All attempts to demonstrate inhibition of submaximal responses of the isolated guinea-pig ileum to histamine by hisperidin, rutin, naringin, and quercitin failed. Evidence has, however, been presented which strongly indicates that the antagonism shown by these glycosides and by the aglycone to the action of histamine on the capillaries of intact animals should be attributed to the formation of active intermediary compounds by metabolic processes (Table IV). Both hisperidin methyl chalcone and the chalcone developed their action

TABLE IV

ME EFFECT RELATIONSHIPS FOR THE ACTION OF SOME FLAVANOID COMPOUNDS ON THE RESISTANCE OF SKIN CAPILLARIES OF MICE TO HISTAMINE

Correction has been made for solvent action in the compilation of this table.

Compound		Route of Injection	Dose/Mouse mg.	Mean Score/Mouse ± the Standard Error (No. of Mice)				
				Control	Time in Min. After Injection			
Name	Nature				10 to 15	20 to 25	30 to 40	55 to 65
Rutin	Glycoside	I.P.	10	20·7 ± 1·26 (10)	19·8 ± 1·34 (10)	16·7 ± 2·58 (10)	13·8 ± 2·13 (10)	13·2 ± 1·78 (10)
Hisperidin	,,	,,	10	21·3 ± 1·08 (10)	21·0 ± 1·27 (10)	15·1 ± 1·76 (10)	12·3 ± 1·82 (10)	12·6 ± 1·44 (10)
Naringin	,,	,,	10	20·4 ± 2·12 (6)	21·3 ± 1·78 (6)	20·7 ± 1·63 (5)	21·2 ± 2·14 (5)	—
Quercitin	Aglycone	,,	10	21·2 ± 1·37 (6)	20·5 ± 2·16 (6)	18·8 ± 2·14 (6)	14·2 ± 2·36 (6)	14·0 ± 2·79 (6)
		I.V.	5	22·9 ± 1·32 (6)	21·8 ± 1·12 (6)	16·4 ± 1·51 (6)	—	—
Hisperidin methyl chalcone	Chalcone	I.P.	5	18·8 ± 1·24 (6)	17·5 ± 0·95 (6)	17·7 ± 1·29 (6)	17·8 ± 1·51 (6)	18·0 ± 1·41 (6)
		I.V.	5	20·7 ± 0·55 (6)	4·5 ± 2·64 (6)	—	15·0 ± 1·61 (6)	—
		,,	2·5	21·9 ± 0·27 (6)	9·0 ± 3·13 (6)	14·8 ± 2·38 (5)	—	—
"Chalcone" (Kodak, Ltd.)	,,	I.P.	10	20·8 ± 2·32 (6)	17·8 ± 2·11 (6)	15·4 ± 1·79 (6)	16·8 ± 2·32 (6)	—
		I.V.	10	21·7 ± 3·31 (5)	12·2 ± 2·24 (6)	14·3 ± 1·70 (6)	—	—
		,,	10	20·6 ± 1·78 (6)	11·5 ± 2·17 (6)	—	—	—

in the whole animal immediately after they had been injected intravenously (Table IV). These compounds therefore required no activation. They were tested for ability to antagonize the action of histamine on smooth muscle both in isolated preparations and in the whole animal. The isolated preparations used were guinea-pig ileum, uterus, and tracheal chain ; these were suspended in oxygenated Tyrode fluid at 32°. Neither chalcone, when added to the bath fluid in concentrations up to 0.1 mg./ml. for periods of contact with the tissue up to 20 min., modified the responses of the tissues to histamine. The intravenous injection of 45 mg./kg. of either chalcone into guinea-pigs anaesthetized with pentobarbitone also failed to alter the pressor responses of the mean arterial pressure to histamine given intravenously. Observations were maintained for 40 min. after the injection of the flavanoid compounds in this last group of experiments.

It was therefore concluded that these flavanoid compounds had no true selective antihistamine action at the dose levels used and that they had antagonized the effects of histamine on the capillaries of the skin solely by their non-specific action on capillary permeability. This type of action was studied by previous authors ; it has been termed, by them, " vitamin-P-like " activity (Bacharach *et al.*, 1942 ; Bourne, 1943 ; Bacharach and Coates, 1944).

The Assay of Flavanoid Compounds for their Capillary Action in Mice.—Both pure flavanoid compounds and extracts of natural products which contained them have been assayed by measurement of the reduction they caused in the sensitivity of the skin capillaries of mice to histamine.

Sixteen 2 by 2 assays have been performed in which rutin in propylene glycol, 50 mg./ml., was used as standard and both standard and test solution were given by intraperitoneal injection. The mean slope of the dose/effect curve in these assays was 15.48. The standard error of this slope, directly determined, was 0.96. Both the number of mice used on each point and the strain of mice used appear to have influenced the precision of these assays. Table V summarizes these observations. In the first seven assays there were 6 mice in each group ; mice from British Drug Houses were used for three and mice from Glaxo Laboratories for four of these assays. Values of b and of s/b have been calculated for each assay. The mean slopes (b) of the dose/effect curves given by the two strains of mice were very similar, but the precision of the assays (s/b) was greater when the B.D.H. strain was employed. The use of 9 or 10 mice in each group further improved the precision of these assays. Overall, irrespective of the strains of mice used, the limits of error ($P=0.95$) obtained with 6 mice in a group averaged $\pm 39.8\%$ of the mean ; the standard error of this average was $\pm 3.11\%$. In the assays in which either 9 or 10 mice were used on each point, the limits of error ($P=0.95$) averaged $\pm 18.93\%$ of the mean, and the standard error of the average was $\pm 2.55\%$. Significant deviation from parallelism of the regression lines for test and standard solutions was not encountered.

TABLE V

VARIATION IN THE PRECISION OF 2 BY 2 ASSAYS OF THE CAPILLARY ACTION OF FLAVANOID COMPOUNDS IN MICE OF DIFFERENT STRAINS

No. of Mice/Group	No. of Assays	Strain of Mice	Regression b ± S.E. (No. Assays)	Range of s/b
6	7	Glaxo	15·1 ± 2·7 (4)	0·32 to 0·51
		B.D.H.	15·3 ± 2·5 (3)	0·17 o 0·35
9 or 10	9	,,	15·7 ± 1·9 (8)	0·07 to 0·21
		Roche	15·8 ± 3·7 (1)	0·19

DISCUSSION

The method presented for the assay of the capillary actions of flavanoid compounds in mice requires reasonable skill and careful attention to timing. The advantages of this method are believed to be speed and economy in space and apparatus. In addition, limits of error are calculable from the internal evidence of each assay.

It may be argued that the doses of flavanoid compounds needed for the purpose of rapid assays (such as 10 mg./30 g. mouse) were so large that these compounds cannot possibly have biological significance. However, their cumulative action has been demonstrated by Bacharach *et al.* (1942), and some fruits are very rich in these compounds. Bacharach and Coates (1944) measured the vitamin-P-like activity of citrus fruits, rose hips, and blackcurrants, and also that of concentrates and purées prepared from these fruits. Their richest source of activity was the peel of citrus fruit. Blackcurrants had the highest potency when this was measured in terms of fresh whole fruit. One hundred grammes of blackcurrants had capillary action equivalent to that of 750 mg. hisperidin. Purées made from this fruit developed further activity in the process and this activity was not destroyed by two years of storage at 0°. These purées developed activity equivalent to 5 g. hisperidin/100 g. of fruit. The possible biological

activity of such natural products cannot therefore be ignored.

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AN INHIBITORY EFFECT OF ACETYLCHOLINE ON THE RESPONSE OF THE GUINEA-PIG ILEUM TO HISTAMINE

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If small doses of histamine are alternated with small doses of acetylcholine on the isolated guinea-pig ileum in Tyrode solution, the response to histamine tends to diminish and may disappear. When the response to the small doses of histamine has disappeared, a contraction may be elicited by larger doses. The diminution is reversible, the response returning if the preparation is left unstimulated by drugs for varying periods of time. This phenomenon also occurs when the loops are suspended in Tyrode solution containing cocaine or atropine.

In the course of experiments with the smooth muscle of the guinea-pig ileum, it was noticed frequently that the response to histamine appeared to be modified after acetylcholine had been used. The present experiments were undertaken to investigate this interaction because it might throw some light on the mechanisms of action of the substances concerned.

Material and Methods

Twenty-five male guinea-pigs, weighing between 300 and 500 g., were used. They were killed by a blow on the head and the lower 15 to 20 cm. of the ileum was removed, washed in normal saline and used immediately. Loops of ileum were tied at both ends and suspended in aerated Tyrode solution in baths of 25 to 30 ml. capacity, at room temperature (23° to 28°). In some experiments, Ringer-Locke solution was used. Contractions were recorded on smoked drums with frontal writing points on isotonic levers.

For every experiment, solutions of histamine acid phosphate (British Drug Houses) and of acetylcholine chloride (Roche) containing in each case 10 μg./ml. of the salt were freshly prepared. Unless otherwise stated, 0.2 ml. of each solution was introduced to the bath.

For most experiments, two loops of bowel from the same guinea-pig were used simultaneously. The control loop was subjected to repeated doses of histamine (2 μg.). The test loop was first subjected to 4 or 5 doses of histamine (2 μg.), and thereafter doses of acetylcholine (2 μg.) were alternated with the doses of histamine.

In 9 experiments, a third loop of bowel was suspended in Tyrode solution containing 1.3 mg. atropine sulphate/l. and was also subjected to alternate doses of histamine and acetylcholine. In 6 further experiments, the third loop of bowel was suspended in Tyrode solution containing 12.5 mg. cocaine/l., and was subjected to alternate doses of histamine and acetylcholine.

In all experiments, after each dose of drug had produced its maximum effect, the tissues were washed twice and the baths refilled with Tyrode solution, and the next drug was added 2 min. later.

Results

In all of 18 experiments it was found that in the loop of bowel which had been subjected to treatment with acetylcholine and histamine alternately the response to histamine rapidly diminished, and in many cases disappeared, while the response to histamine in the control loop remained unchanged (Fig. 1). The rapidity with which this decline occurred varied from animal to animal. In some cases the histamine response began to diminish after two or three doses of acetylcholine. In others it diminished only after 30 or 40 doses. In those instances in which the histamine response did not disappear after 5 or 6 doses of 2 μg. of acetylcholine, increasing the doses of acetylcholine to 4 μg. or 6 μg. or more produced a diminution in the response to histamine after several further doses. In all cases the response to acetylcholine in the test loop was maintained throughout the experiment.

In six experiments when the histamine response had disappeared in the test loop, the experiment was interrupted and the loops were left in fresh Tyrode solution for varying periods of time (10 to 90 min.). On resuming the experiment, the histamine response of the test loop was found to have returned to a greater or lesser extent the contrac-

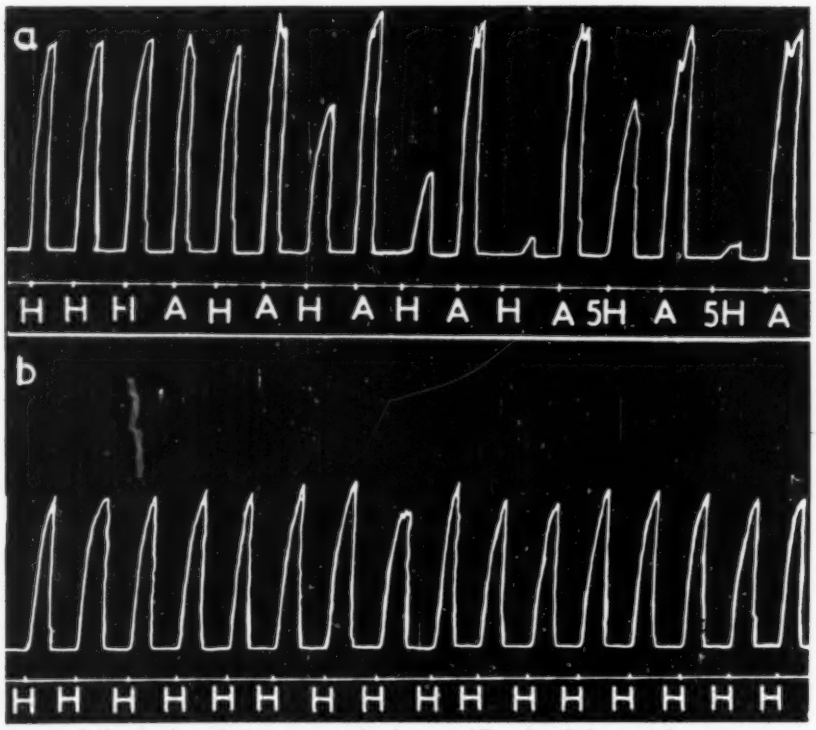

FIG. 1.—Isolated guinea-pig ileum preparation in aerated Tyrode solution at 24°. *a*, Test loop; *b*, control loop. Doses of drugs were administered at intervals of approximately 3 min. Bath volume 25 ml. H=2 μg. of histamine; 5H=10 μg. of histamine; A=2 μg. of acetylcholine. (Tracing retouched.)

tions in some cases being as marked as at the start of the experiment. When repeated doses of acetylcholine were again administered the histamine response once again diminished.

In six experiments after the disappearance of the histamine response, a larger dose of histamine (10 μg.) was then introduced. In each instance a contraction was obtained which in some cases was equivalent to the initial histamine contraction (Fig. 1). This response, however, also diminished after several further doses of acetylcholine.

The Effect of Atropine.—As expected, atropine added to the Tyrode solution prevented any response to acetylcholine. In such atropinized preparations acetylcholine, given alternately with histamine, failed to diminish the response to histamine. In some experiments a decrease in the reaction to histamine occurred following the first few doses of acetylcholine, but the response to histamine eventually returned to the original amplitude.

The Effect of Cocaine.—The presence of cocaine in a concentration of 1:80,000, believed to depress the neurones but not the muscle cells (Feldberg and Lin, 1949a), did not make any significant difference to the diminution of the histamine response following acetylcholine administration.

Discussion

One point of interest which these experiments demonstrate is that an "antagonism" between two drugs acting on intestinal muscle *in vitro* may not be immediately obvious when the drugs are first administered and may, in fact, only emerge when both drugs have been used repeatedly on the tissue.

According to the work of Ambache and Lessin (1955) in which type D botulinum toxin was used to analyse the types of action of various intestinomotor drugs, both acetylcholine and histamine act mainly directly on the muscle. From the present experiments it is evident that, under the conditions obtaining, acetylcholine when administered repeatedly in fairly high doses alters the reactivity of the muscle to histamine. As this effect occurs also in cocainized ileum but not in atropinized ileum, it would seem that neuronal elements are not primarily involved, and that it is the muscle itself which undergoes a change in sensitivity.

Further factors of note are that the change in sensitivity to histamine occurs only after repeated administration of acetylcholine; that it can almost always be produced provided sufficiently large concentrations of acetylcholine are used, and that this altered sensitivity to histamine is temporary and can be reversed by subsequently withholding acetylcholine.

The explanation of the effect is not clear, but the following possibilities might be considered.

(1) Acetylcholine may be an antagonist of histamine. This antagonism may not normally be demonstrable because acetylcholine itself contracts the muscle. However, after the acetylcholine stimulation has passed off the antagonistic effect may become apparent. This hypothesis is supported indirectly by the finding (Schild, 1947) that atropine is an antagonist of histamine on the guinea-pig ileum. Just as acetylcholine and atropine both react with acetylcholine

receptors, they might also both react, with a weaker affinity, with histamine receptors. The lack of depression by acetylcholine in the atropinized preparation might then be explained on the grounds that atropine and acetylcholine occupy the same sites on the histamine receptors so that, when they are blocked by atropine, they cannot be further blocked by acetylcholine.

(2) A different explanation is based on the suggestion (Burn, 1950 ; Feldberg, 1950) that acetylcholine may be a " local hormone " in some tissues. It has been established by several workers that acetylcholine is continuously synthesized in the wall of the ileum. The work of Feldberg and Lin (1949b) seemed to indicate that this synthesis could not be attributed only to the nervous elements. If this theory is correct, it might be expected that, in an ileum preparation set up in an isolated organ bath under slight tension but not contracting, there would still be a low level of synthesis and breakdown of endogenous acetylcholine. The possibility is suggested here that such a process, while not in itself causing contraction, might be the basis for the capacity of the muscle to respond to a stimulant such as histamine. Repeated doses of acetylcholine might tend, by the law of mass action, to depress the endogenous synthesis of acetylcholine, so that subsequent administration of histamine would have less and less effect until the response would disappear altogether. That exogenous acetylcholine has such a depressant effect on endogenous acetylcholine synthesis has been shown in the heart (Bülbring and Burn, 1949 ; Burn, 1956).

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THE BIOLOGICAL ACTION OF CHLOROPHENOLS

BY

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The biological action of a series of chlorinated phenols has been investigated. With increasing chlorination there is an increase in toxicity in which the convulsant action of phenol is replaced by the signs characteristic of poisoning by dinitrophenol; the higher chlorinated phenols produce a contracture of the isolated rat phrenic nerve diaphragm and a stimulation of *in vitro* oxygen uptake of rat brain homogenate. These actions of the chlorinated phenols have been correlated with their dissociation constants; it is suggested that the higher chlorinated phenols interfere with oxidative phosphorylation, and that this property may be attributed to the chlorophenate ion. The convulsant action of the lower chlorinated phenols is probably associated with the undissociated molecule.

Chlorination of phenol produces a series of nineteen compounds comprising mono-, di-, tri-, and tetrachloro- isomers, and one pentachloroform. All are crystalline solids at room temperature with the exception of *ortho*chlorophenol which is a liquid. The lower compounds in the series are employed medically for their powerful antiseptic properties which, in many cases, are greater than those of phenol itself (von Oettingen, 1949). Tetra- and penta-chlorophenols are important wood preservatives, while the latter is also used as a herbicide, fungicide, insecticide, and molluscicide. These uses of the higher chlorophenols have been reviewed by Truhaut, Vitte and Boussemart (1952).

On account of the differences in test animals and injection techniques employed by authors, including the fact that only seven chlorophenols appear to have been studied, it is difficult to trace the way in which chlorination affects the toxicity of phenol (Bechold and Ehrlich, 1906; Binet, 1896; Deichmann, 1943; Kehoe, Deichmann-Gruebler, and Kitzmiller, 1939). The general effect of chlorination is to increase the toxicity while reducing the convulsant activity characteristic of phenol. Pentachlorophenol is non-convulsant and is characterized by its stimulant effect on oxidative processes, exhibited both *in vitro* and *in vivo*, which is very similar to the activity of dinitrophenols as shown by Weinbach (1954).

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Employing eleven chlorophenols as well as phenol itself, a systematic study has been made of the quantitative changes in toxicity occurring with progressive chlorination of phenol. Median lethal doses have been taken as the basis for comparison and have been determined under strictly controlled conditions. An attempt has been made to correlate these quantitative changes in toxicity with changes in the qualitative nature of the effects, and also with the action on oxidative processes studied *in vitro*. In turn, these changes have been considered in conjunction with the dissociation constants of the phenols.

The effect of the progressive chlorination of phenol upon the isolated rat diaphragm has also been examined.

METHODS AND MATERIALS

Acute Toxicity Tests.—All toxicity tests were carried out on male albino rats, weighing 125 to 175 g., which had been fasted for 24 hr. Experiments on rats which had received food prior to dosing showed a considerably lower toxicity.

Median lethal doses were determined by injecting the chlorophenols intraperitoneally in 10 ml. olive oil/kg. body weight. Using the method of moving averages (Thompson, 1947), successive doses were spaced by a factor of 2.0, four rats being injected at each dose level. Fiducial limits for $P=0.05$ were calculated by the method of Weil (1952). All injections were carried out at a room temperature of $23° \pm 1°$.

Enzyme Experiments.—The effects of chlorophenols on the oxygen uptake of rat brain homogenate were

studied by means of the Warburg manometric method. The chlorophenols were added to the flasks as chlorophenates. Stock solutions, prepared by dissolving the chlorophenols in equivalent volumes of N-NaOH, were diluted in substrate-buffer; 0.5 ml. of these solutions was added to the side-arms of the flasks to give final flask concentrations ranging from 10^{-6} to 10^{-3} M. In control flasks, substrate-buffer alone was added. For absorbing carbon dioxide produced, 0.2 ml. of 20% potassium hydroxide and filter papers were added to the central wells.

For preparing the enzyme, the method adopted was essentially that of Tyler (1950). Using an all-glass hand homogenizer, a 10% homogenate of rat cerebrum was prepared in Krebs-Ringer phosphate (0.154 M, *p*H 7.4) containing 10^{-2} M-glucose (Umbreit, Burris, and Stauffer, 1949). The homogenizer was immersed in an ice-water mixture. After straining the homogenate through gauze, 1.0 ml. was added to the main compartment of each flask together with 0.5 ml. buffer. The flasks were set in the water bath at 38° within 20 min. of the rats being killed. The atmosphere in the flasks was air. After temperature equilibration for 15 min. followed by control readings for 30 min., the contents of the side-arms were tipped in and readings then taken 6 and 36 min. later for measuring the oxygen uptake. These 30 min. oxygen uptakes were compared with those of control flasks run concurrently.

Rat Phrenic Nerve-diaphragm Preparation.—Preparations were set up according to the method of Bülbring (1946), in Tyrode solution containing 0.2% glucose and aerated with 5% CO_2 in oxygen. Supramaximal, rectangular wave stimuli of 0.2 m.sec. duration were applied to the nerve at the rate of 7/min. The capacity of the bath was 60 ml. Sodium chlorophenates were added to the bath in doses of 1 to 200 μ moles. At the time of testing, 3:4:5- and 2:3:6-trichlorophenols were not available.

Relationships between the different variables were estimated by calculation of co-efficients of correlation (Snedecor, 1948).

Results

Median Lethal Doses.—Median lethal doses are presented in Table I. With doses around these values, death usually occurred within 1 hr. of injection; the highest doses caused death within 5 to 15 min. No further deaths occurred after 3 hr.

Convulsions.—Injections of phenol, the monochlorophenols, 2:6-dichlorophenol, and 2:4:6-trichlorophenol were all followed by the same syndrome. Occasionally there was an initial brief bout of excited behaviour with rapid running, or vigorous nose-rubbing. Tremors appeared in 40 to 120 sec. and almost immediately became generalized; sometimes the head and forepaws were seen to be affected first. When poisoning was sufficiently great for the tremors to increase in severity, intermittent convulsions developed and the rat usually fell on its side with loss of righting reflexes. When recovery followed, the rats were sluggish and hypotonic for several hours. If poisoning was fatal, coma and dyspnoea set in. Rats injected with 2:3:6-trichlorophenol often convulsed violently when handled, but otherwise lay prostrate with hypotonia.

Hypotonia.—All compounds proved capable of producing hypotonia although this was difficult to detect at first after injections of the convulsant phenols. When the convulsions lessened, however, the hypotonia became obvious and persisted for several hours.

After injecting 2:4-dichlorophenol, 2:3:6-, 3:4:5-, and 2:4:5-trichlorophenols, tetrachlorophenol, or pentachlorophenol, hypotonia was observed in the hind-limbs in 2 to 3 min. This gradually progressed to involve the forelimbs and neck so that the rat was completely prostrate. The

TABLE I

ACUTE TOXICITIES OF CHLOROPHENOLS DETERMINED BY INTRAPERITONEAL INJECTION IN OLIVE OIL INTO MALE ALBINO RATS

The rats weighed 125 to 175 g.

Position of Chlorine Atoms in Phenol	*p*K	Median Lethal Dose			Convulsant Activity	Maximal Change in Rectal Temperature (° C.)	Rigor Mortis within 5 min. of Death
		mg./kg.	Limits of Error ($P=0.05$)	m. moles/kg.			
—	9·98	250	(188–333)	2·66	+	−2·5	−
4	9·37	281	(188–419)	2·18	+	−2·5	−
3	9·12	355	(266–473)	2·75	+	−2·5	−
2	8·65	230	(—)	1·78	+	−2·0	−
2:4	7·85	430	(186–997)	2·64	(Occasional twitches)	−0·5	−
3:4:5	7·83	372	(220–630)	1·88	−	+0·5	+
2:4:5	7·07	355	(—)	1·79	−	+0·5	+
2:6	6·91	390	(—)	2·39	+	−0·7	−
2:4:6	6·62	276	(185–412)	1·40	+	+0·5	+
2:3:6	5·98	308	(161–591)	1·56	(Rats sometimes convulsed when handled)	+0·5	+
2:3:4:6	5·46	130	(97–173)	0·56	−	+4·0	+
2:3:4:5:6	5·00	56	(38–84)	0·21	−	+4·5	+

eye reflex weakened and there was no withdrawal response to toe pinching. Muscle twitches seldom occurred spontaneously nor could they be evoked by auditory and mechanical stimuli except in the instance of 2:4-dichlorophenol.

Body Temperature.—The rectal temperature of control rats ranged from 37.7 to 38.3°. Injection of penta- and tetra-chlorophenols caused marked rises in temperature, while the trichlorophenols were either without effect, or caused a slight elevation. Phenol and the mono- and di-chlorophenols caused either no change or a slight decrease in rectal temperatures. These effects are indicated in Table I.

Respiratory Effects.—Respiration was at first accelerated but became slower and dyspnoeic as coma developed, particularly with tetra- and penta-chlorophenols. The extremities became cyanosed and when coma set in poisoning was usually fatal; asphyxial spasms occurred finally about 30 sec. before death. In tetra- and penta-chlorophenol poisoning, respiration always ceased ½ to 2 min. before the heart stopped beating. With the other compounds, respiration ceased either just before the heart or simultaneously with it.

Rigor Mortis.—In control rats killed by ether, rigor mortis occurred 50 min. after death. Stiffening always occurred first in the neck; stiffening of the rest of the spinal muscles often did not occur for a further 30 min. Death due to penta- and tetra-chlorophenols or to 2:4:6- or 2:4:5-trichlorophenols was followed by rapid onset of intense rigor mortis within 3 to 5 min. Rapid appearance of a somewhat weaker rigor occurred after fatal poisoning with 3:4:5- and 2:3:6-trichlorophenols. In poisoning due to the former compounds, rigor often preceded death, normally commencing in the jaws which were clamped down upon a deeply cyanosed tongue for about 1 min. before the trunk began to stiffen and the respiratory movements ceased. Fatal poisoning with phenol and with the other chlorophenols was also followed by onset of rigor mortis rather earlier than in controls; this applied particularly to the higher dose groups.

Other Effects.—Chromodacryorrhoea, lachrymation, salivation and diarrhoea were sometimes produced. Macroscopic haematuria was observed in no case, although it has been reported to follow subcutaneous injection of monochlorophenols (Binet, 1896).

Enzyme Experiments.—The results of the experiments on the oxygen uptake of brain homogenate are shown in Table II. Phenol and *o*-chlorophenol caused no stimulation of oxygen uptake at concentrations from 10^{-6} to 10^{-3} M; phenol, in fact, caused slight depression of oxygen consumption. At concentrations between 2.5×10^{-5} and 10^{-3}M, *p*- and *m*-chlorophenols and 2:6-dichlorophenol caused slight stimulation of oxygen uptake; it is probable that the peak stimulation concentrations lie above 10^{-3} M. 2:4-Dichlorophenol, the four trichloro- isomers, tetra- and penta-chlorophenols all stimulated oxygen uptake at relatively low concentrations while depressing the uptake at higher concentrations.

Isolated Diaphragm Preparation.—Five of the compounds tested were found to send the contracting diaphragm into contracture. Minimum doses, which when added to the bath elicited this effect, were of the order of 1 to 5 μ moles for tri-, tetra-, and penta-chlorophenols, but 20 μ moles for 2:4-dichlorophenol. With relatively low doses the contracture was often preceded by a brief potentiation

TABLE II

EFFECT OF CHLOROPHENATES ON OXYGEN CONSUMPTION OF RAT BRAIN HOMOGENATE AND ON THE TWITCH RESPONSE OF ISOLATED RAT DIAPHRAGM TO INDIRECT STIMULATION

+ Indicates potentiation. The numerals in parentheses indicate dose added to 60 ml. bath. The rat brain homogenate was incubated with 10^{-2}M-glucose at 38°, *p*H 7·4.

Position of Chlorine Atoms in Phenol	*p*K	% Ionization at *p*H 7·4	Oxygen Uptake			Muscle Twitch Response
			Peak Stimulation Conc. (M)	Equivalent Anionic Conc. (M)	Peak Stimulation % above Normal	
—	9·98	0·25	—	—	None	+ (10 μ moles)
4	9·37	1·0	$\leqslant 10^{-3}$		47 at 10^{-3} M	+ (10 μ moles)
3	9·12	2·0	$\leqslant 10^{-3}$		50 at 10^{-3} M	+ (10 μ moles)
2	8·65	5·3	—	—	None	(−) (10 μ moles)
2 : 4	7·85	26·2	2.5×10^{-4}	6.5×10^{-5}	70	Contracture (20 μ moles)
3 : 4 : 5	7·83	27·3	10^{-4}	2.7×10^{-5}	112	Not tested
2 : 4 : 5	7·07	68·2	5×10^{-5}	3.4×10^{-5}	120	Contracture (5 μ moles)
2 : 6	6·91	75·7	$\leqslant 10^{-3}$		26 at 10^{-3} M	(+) (10 μ moles)
2 : 4 : 6	6·22	94·0	10^{-4}	9.4×10^{-5}	92	Contracture (5 μ moles)
2 : 3 : 6	5·98	96·5	5×10^{-5}	5×10^{-5}	38	Not tested
2 : 3 : 4 : 6	5·46	99·0	5×10^{-5}	5×10^{-5}	154	Contracture (1 μ mole)
2 : 3 : 4 : 5 : 6	5·00	99·5	5×10^{-5}	5×10^{-5}	200	Contracture (1 μ mole)

of the twitch amplitude. Addition to the bath of 10 to 100 μ moles of *m*- or *p*-monochlorophenols caused powerful potentiation of the twitch which was not followed by contracture. Addition of 10 μ moles *o*-chlorophenol, on the other hand, caused a slight depression, while 10 to 50 μ moles phenol caused potentiation.

Discussion

Progressive chlorination of phenol increases the acidity of the phenolic hydroxyl- group which is demonstrated by the decrease in *p*K value with increasing chlorination as displayed in Table I. All the *p*K values in this table were determined by a potentiometric titration procedure which has been described in detail elsewhere (Gage, 1957); they do not differ greatly from values published in the literature by other methods.

It is evident from Table I that as phenol is progressively chlorinated the molar toxicity shows a consistent tendency to increase when the *p*K value falls below 7.0. These more toxic chlorophenols are more than 90% ionized at physiological *p*H values. The decrease in *p*K value with increasing chlorination is also accompanied by a change in the effects elicited. As convulsions are undoubtedly the most characteristic effect of chlorophenols with *p*K values of 8.65 or higher, it would seem that they can be attributed to the undissociated molecules. Convulsions are not typical of chlorophenols with lower *p*K values, although they are produced by 2:6-dichlorophenol, 2:4:6-trichlorophenol, and sometimes by 2:3:6-trichlorophenol. It is noteworthy that these three compounds are all substituted in the di-*ortho*- position; the significance of this will be mentioned later. Chlorophenols with *p*K value of 7.85 or less appear to be associated with the production of marked hypotonia without convulsions, an increase in body temperature, and an early onset of rigor after death; these effects may, therefore, be attributed to the chlorophenate ion.

The higher chlorinated phenols are also associated with an ability to cause contracture of the isolated diaphragm, and to stimulate oxygen uptake *in vitro*. The manner in which oxygen uptake is stimulated by chlorophenols and dinitrophenols is not fully understood, but it seems likely that these substituted phenols have a common mode of action. As was originally shown by the *in vitro* studies of Loomis and Lipmann (1948) and of Weinbach (1954), the dinitrophenols and pentachlorophenol are inhibitors of oxidative phosphorylation. These compounds dissociate the process of oxygen uptake from the synthesis of high energy phosphate bonds, processes which are normally interdependent. While phosphorylation is inhibited, oxygen consumption may, with the appropriate enzyme system, be shown to be greatly accelerated. If the increase of oxygen uptake observed in our experiments is the outcome of inhibition of oxidative phosphorylation, then it may well be that all the chlorophenols which stimulate oxygen uptake inhibit phosphorylation in corresponding degree, the effect being due to chlorophenate ions.

The results of enzyme experiments indicate that, with the exception of 2- and 2:6-dichlorophenol, the ability to stimulate oxygen uptake increases with progressive chlorination of phenol. Thus, with increasing acidity of the compounds, the molar concentration of chlorophenate required to produce peak stimulation of oxygen uptake decreases, and the actual height of the peak stimulation above normal increases. Taking into account the *p*K values and % ionization at *p*H 7.4 of the buffered medium, the molar concentrations of chlorophenate ions which produced peak stimulation have been calculated. As indicated in Table II, these concentrations lay between 2.7×10^{-5} M and 9.4×10^{-5} M. This suggests that for the particular enzyme system employed, peak stimulation of oxygen uptake, regardless of its actual height above the normal level, is produced by a fairly constant concentration of chlorophenate ions. The significance of this concentration of anions may be that it corresponds to the concentration of some factor in the homogenate affected by these ions. Using the same enzyme system, Tyler (1950) found that 2:4-dinitrophenol, which has a *p*K value of 4.1, also produced maximum stimulation at a concentration of 5×10^{-5} M. It is suggested that the differing degrees of peak stimulation are due to the chemical peculiarities of the chlorophenate (or nitrophenate) ions causing various rates of interaction with the postulated factor. This explanation is in harmony with current theories of the effect of dinitrophenols on oxidative phosphorylation (Lardy and Wellman, 1953; Lee and Eiler, 1953). These theories postulate the existence of a labile, phosphorylated intermediate whose rate of continual synthesis and breakdown determines the rate of oxygen consumption. The substituted phenols are thought to compete for combination with the precursor of this intermediate, forming a compound of greater instability; the synthetic and breakdown processes are thereby accelerated causing an increased rate of oxygen uptake.

The anomalous position of 2:6-dichlorophenol in Table II recalls the observations of Blackman,

Parke, and Garton (1955), who found that chlorophenols substituted in these positions deviate from the linear relationship which they have shown to exist between *p*K value and the ratio of standard bactericidal activity to solubility. It seems probable that the anion produced by ionization of 2:6-dichlorophenol cannot react, presumably with some basic centre in the receptor system, by virtue of the steric or electronic interference by the ortho chlorine atoms.

It is interesting to notice the fairly close negative correlation between median lethal doses and the ability to stimulate oxygen uptake *in vitro*. For the statistical tests of correlation, stimulation of oxygen uptake was taken as the % stimulation above the normal level produced by 10^{-5} M chlorophenate. The coefficient of correlation was -0.86 on comparison of % increase in oxygen uptake with median lethal dose.

As shown by several authors (Weeks and Chenoweth, 1952; Judah and Magee, 1954; Barnes, Duff, and Threlfall, 1955), 2:4-dinitrophenol sends the isolated rat diaphragm into contracture, this being accompanied by profound falls in the contents of creatine phosphate and adenosine triphosphate in the muscle. This production of contracture may be attributable to the ability of 2:4-dinitrophenol to inhibit oxidative phosphorylation. It is, therefore, not surprising to find that pentachlorophenol, another inhibitor of oxidative phosphorylation, is able to cause the same effect. Production of contracture by other chlorophenols also suggests that these compounds are inhibitors of oxidative phosphorylation which would accord with their ability to stimulate oxygen uptake (see Table II). In the present experiments, contracture was preceded by slight potentiation of the twitch response; an effect which was not reported by Barnes *et al.* (1955) for 2:4-dinitrophenol. Unfortunately, no explanation can be given for the potentiation of the twitch response produced by phenol, *p*- and *m*-monochlorophenols, and by 2:6-dichlorophenol. Mogey and Young (1949), who studied the antagonistic action of phenolic substances on the effect of tubocurarine on the rat diaphragm, found that phenol and *p*-chlorophenol at a concentration of 10^{-4} M caused only 1 to 7% inhibition of rat brain cholinesterase activity *in vitro*. It seems highly unlikely, therefore, that potentiation of the twitch is due to the anticholinesterase activity of these compounds.

The appearance of rapid, or even of premature, rigor mortis is also found in dinitrophenol poisoning (Hall, Field, Sahyun, Cutting, and Tainter, 1933; Parker, 1954; Parker, Barnes, and Denz, 1951) and it has been suggested there may be a common mechanism underlying this rapid rigor mortis and contracture of the isolated diaphragm (Barnes *et al.*, 1955). Further evidence for the association between these two effects is provided by the present work since four of the chlorophenols shown to cause contracture of the isolated diaphragm also caused rapid rigor mortis (Tables I and II).

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THE ACTION OF NITROPHENOLS ON THE METABOLIC RATE OF RATS

BY

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The effect of the mono- and di-nitrophenols and certain related compounds has been determined on the rate of oxygen consumption, the rate of carbon dioxide output and the rectal temperature of the Wistar albino rat.

Of the compounds examined, only 2: 4-dinitrophenol and its derivative, 3: 5-dinitro-*o*-cresol, stimulated metabolic rate. 2-Nitrophenol and 2: 3-, 2: 6-, and 3: 5-dinitrophenol produced no change in metabolic rate; 3-nitrophenol and 2: 5-dinitrophenol had no action on carbon dioxide production although they caused a decrease in oxygen consumption. 4-Nitrophenol and 3: 4-dinitrophenol increased only the rate of carbon dioxide output; 2-amino-4-nitrophenol increased the rate of carbon dioxide output and decreased the rate of oxygen consumption ; 4-amino-2-nitrophenol caused depression of metabolic rate.

It was confirmed that neither rectal temperature nor carbon dioxide output could replace rate of oxygen consumption as a reliable index of metabolic stimulant action. An apparatus is described which facilitates measurement of the oxygen consumption of small mammals.

The actions of the mono- and di-nitrophenols on metabolic rate are obscure ; previous investigators reached inconsistent conclusions. 3-Nitrophenol, for example, is described in turn as a rectal temperature depressant (Gibbs and Reichart, 1894), without action on rectal temperature (Magne, Mayer and Plantefol, 1932 ; Tainter, Bergstrom and Cutting, 1935), a stimulant of yeast respiration (Field, Martin and Field, 1935) and a stimulant of Arbacia egg respiration (Clowes and Krahl, 1937 ; Tyler and Horowitz, 1937). Similarly, 2: 5-dinitrophenol has been reported as a rectal temperature depressant (Magne *et al.*, 1932), without action on rectal temperature (Tainter *et al.*, 1935), a depressant of yeast respiration (Genevois and Saric, 1932) and a depressant of oxygen consumption of dogs (Magne *et al.*, 1932).

The present investigation was undertaken to re-examine systematically the effect of the mono- and di-nitrophenols and three related compounds on the metabolic rate of the intact mammal. In the course of the work an attempt was made to establish a practical method for determining the metabolic rate of small mammals.

The investigation was carried out in two stages. Each nitrophenol was initially examined for action on carbon dioxide output, an index recommended by Benedict and Homans (1911) for preliminary metabolic studies, and for effect on rectal temperature. The action of each compound on rate of oxygen consumption was then determined.

METHODS

Carbon Dioxide Output.—The apparatus was based on the open-chain circuit of Haldane (1892). Ventilation was by a centrifugal blower (Thermotank Ltd.) with a maximum output of 22,000 cu. ft./hr., driven by a $\frac{1}{4}$ h.p., 1,425 rev./min., continuously-rated electric motor. The air was led from a 3.5 l. plenum box, through soda-lime towers, to a copper manifold with outlet pipes of $\frac{1}{2}$ in. diameter. The animal chambers were Kilner jars, 800 ml. capacity ; metal lids fitted with rubber gaskets and two copper pipes of $\frac{1}{8}$ in. diameter replaced their usual glass tops. The absorption trains were three glass tubes (12 in. by 1 in.) connected in series ; the first and last tubes were filled two thirds with 8 to 14 mesh calcium chloride granules and one third with magnesium perchlorate ; the middle tube contained small grain "Indicarb" soda-lime.

In each run, 6 male and 6 female rats of 160 to 200 g. body weight were used ; 2 were controls and 10 were treated. Each rat was weighed, treated as required and placed in the animal chamber, which was then connected to a manifold outlet. The chambers were ventilated for 10 min., the weighed absorption trains connected and the effluent passed through

the trains for 1 hr. The absorption tubes were then removed, the rectal temperature of the rats taken and the tubes re-weighed. The weight increase in the last two tubes was the weight of CO_2 absorbed.

Oxygen Consumption.—A manometric air-filled apparatus was constructed. The circuit (Fig. 1 (*b*)) consisted of a gas-tight reservoir (R), animal chamber (A) and soda-lime container (S) ventilated by a pump (P) with an open manometer (M) attached between pump and reservoir. The reservoirs were 45 l. steel drums, to which connecting pipes of $\frac{3}{8}$ in. copper tubing were fixed by brazing to a $\frac{1}{4}$ in. steel plate, which was in turn soldered to the end of the drum with a wiped joint. Kilner jars with modified lids were used for the animal chamber and soda-lime container. The pump (C. F. Palmer Ltd.) was an electrically-driven reciprocating rubber bellows respiration pump with an output of $3\frac{1}{2}$ l./min. at 40 strokes/min., rendered gas-tight by careful reassembly, replacing the original rubber gaskets with neoprene gaskets. Connexions were made with glass Y-tubes, heavy rubber tubing and $\frac{3}{8}$ in. copper tubing. A

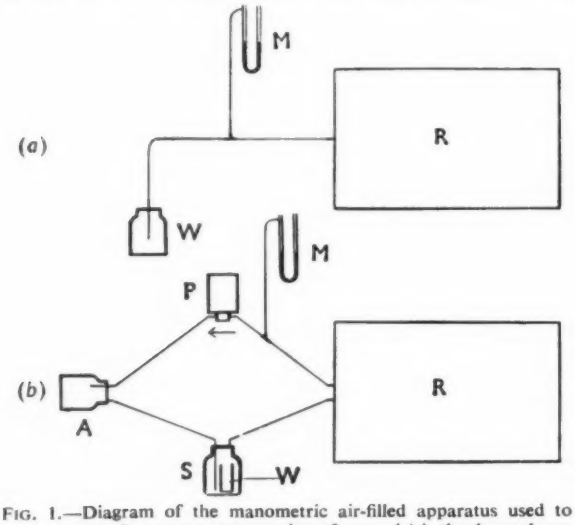

FIG. 1.—Diagram of the manometric air-filled apparatus used to measure the oxygen consumption of rats. (*a*) is the thermobarometer, (*b*) the experimental system. R, 45 litre reservoirs; M, manometers; P, pump; A, animal chamber; W, water containers; S, soda-lime jar.

second reservoir with manometer was set up as a thermobarometer (Fig. 1 (*a*)) to correct for variations in ambient conditions. The manometers, graduated in mm., were filled with Brodie-Krebs fluid (Krebs, 1951). Containers with 20 ml. water (W) were introduced into both systems to stabilize the contribution of water vapour pressure to the internal pressure.

The weighed rat was introduced into the animal chamber, both manometer taps closed, and the pump operated for 5 min. with the circuit open between pump and reservoir, to ventilate the entire system. A pressure of about 120 mm. fluid was built up in the reservoir by blocking the air outlet, the circuit reconnected and the airtightness of the apparatus checked. The pump was then operated for 10 min. to establish equilibrium, before taking the first reading of the room temperature thermometer and manometers with the pump in the closed position. Subsequent readings of thermometer and manometers were made at 15 min. intervals for 1 hr. Multiplication of the corrected pressure decrement by the calibration constant, k, gave the oxygen consumed (ml./hr.) at N.T.P. k was evaluated by calibration of the apparatus from $k=273V/TP_o$, where V=gas volume (ml.), T=room temperature (°A.), P_o=10,025 mm.; V was determined by the method of Gerhartz (1926).

Room temperature was maintained at 18 to 20° in all experiments.

The nitrophenols were dissolved in 0.1N-NaOH and administered intraperitoneally to the rats as solutions of *p*H 7 to 9.

RESULTS

Carbon Dioxide Output.—Six estimates of carbon dioxide output and rectal temperature were obtained with each of 4 doses of the drug, from an arbitrarily selected low dose to the maximum dose tolerated by most rats under the experimental conditions. Individual estimates were rejected if the rat convulsed or died during the experiment, if less than 0.30 g. carbon dioxide was absorbed in 60 min. except when respiratory movement was barely perceptible, or if the rat was unduly restless.

The action of each nitrophenol on carbon dioxide output was established by testing the hypothesis that there is a between-dose component of variance in carbon dioxide output; if the null hypothesis was rejected, the regression of carbon dioxide output on dose was calculated, and its significance tested. In each instance it was found that, where there was significant regression, it was linear. Table I summarizes the results of experiments in which the null hypothesis was accepted, Table II where it was rejected. Fig. 2 shows the

TABLE I

ANALYSES OF VARIANCE OF RATE OF CO_2 OUTPUT OF RATS RECEIVING NITROPHENOLS, WHERE THERE WAS NO SIGNIFICANT BETWEEN-DOSE VARIANCE

Within-dose variance was accepted as homogeneous when Bartlett's test gave $P>0.05$. Measurements were made at 4 doses within the given range, and 6 estimates of CO_2 obtained for each dose.

Compound	Dose Range (mg.)	*P* Bartlett's Test	Mean Squares	
			Between Doses d.f.=3	Within Doses d.f.=20
2-Nitrophenol ..	80–120	>0·50	0·0096	0·0065
3- ,, ..	30–45	>0·05	0·0063	0·0047
2 : 3-Dinitrophenol ..	15–24	>0·50	0·0138	0·0054
2 : 5- ,, ..	10–25	>0·50	0·0030	0·0054
2 : 6- ,, ..	1–6·5	>0·10	0·0037	0·0054
3 : 5- ,, ..	2–7	>0·50	0·0033	0·0057
4-Amino-2-nitrophenol	10–50	>0·10	0·0037	0·0017

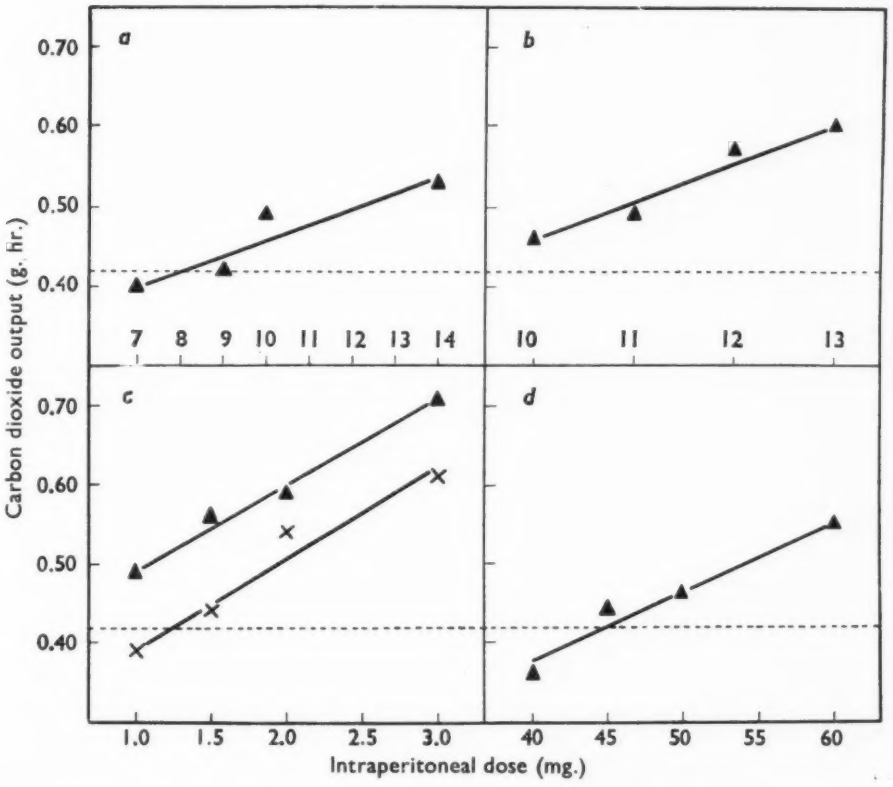

FIG. 2.—The relation between the CO_2 output of Wistar albino rats and intraperitoneal dose of (*a*) 4-nitrophenol, (*b*) 3 : 4-dinitrophenol, (*c*) 2 : 4-dinitrophenol (▲) and 3 : 5-dinitro-*o*-cresol (X), and (*d*)2-amino-4-nitrophenol. The dotted lines represent the mean carbon dioxide output of 96 control rats.

regression lines of dose on carbon dioxide output for 4-nitrophenol, 3:4-dinitrophenol, 2:4-dinitrophenol, 3:5-dinitro-*o*-cresol, and 2-amino-4-nitrophenol.

4-Amino-2-nitrophenol had a peculiar effect. An irregular decrease in carbon dioxide output occurred, with a mean of 0.33 g./hr. for the 24 observations, but, although the difference between this mean and that of control rats was highly significant according to the *t* test ($P<0.001$), no significant between-dose component of variance was found.

The carbon dioxide outputs of 96 control rats had an apparently normal distribution, with a mean of 0.42 g./hr. and standard deviation 0.05. In the course of these experiments a quality control chart was used, and confirmed that the variables were in statistical control.

Rectal Temperature.—The effect of each nitrophenol on rectal temperature was determined by testing the significance of the difference between the mean temperature of the 6 rats which received the maximum non-fatal dose and the grand mean temperature of control rats. The rectal tempera-

TABLE II

ANALYSES OF VARIANCE OF RATE OF CO_2 OUTPUT OF RATS RECEIVING NITROPHENOLS, WHERE THERE WAS A SIGNIFICANT BETWEEN-DOSE VARIANCE

Linear regression of CO_2 output upon dose was established in each instance; the regression equations are shown. Within-dose variance was accepted as homogeneous when Bartlett's test gave $P>0.05$. Measurements were made at 4 doses within the given range, and 6 estimates of CO_2 output obtained for each dose.

Compound	Dose Range (mg.)	*P* Bartlett's Test	Mean Squares: Due to Regression d.f. = 1	Mean Squares: Deviation from Regression d.f. = 2	Mean Squares: Within Doses d.f. = 20	Regression Equation x = Dose (mg.) y = CO_2 Output (g./hr.)
4-Nitrophenol	7–14	>0·05	0·0585	0·0032	0·0035	$y=0.0194x+0.27$
2 : 4-Dinitrophenol	1– 3	>0·10	0·1585	0·0007	0·0033	$y=0.1100x+0.38$
3 : 4- "	10–13	>0·10	0·0686	0·0016	0·0053	$y=0.0478x-0.02$
3 : 5-Dinitro-*o*-cresol	1– 3	>0·10	0·1801	0·0040	0·0051	$y=0.1171x+0.27$
2-Amino-4-nitrophenol	40–60	>0·50	0·0986	0·0024	0·0021	$y=0.0087x+0.03$

tures of 90 control rats were apparently normally distributed, with a mean temperature of 100.3° F. and standard deviation 0.8. Table III shows that the mean temperatures of rats receiving all but 3:4- and 3:5-dinitrophenol are unlikely to be drawn from a control population. It was concluded that only 2:4-dinitrophenol and 3:5-dinitro-*o*-cresol increased rectal temperature; the

TABLE III

MEAN RECTAL TEMPERATURES OF GROUPS OF SIX RATS RECEIVING THE MAXIMUM NON-FATAL DOSE OF NITROPHENOLS

P is the probability, by the *t* test, that there is no significant difference between the mean temperature of each group of treated rats and the grand mean control temperature (100·3° F.).

Compound	Dose (mg.)	Mean Temperature (° F.)	*P*
2-Nitrophenol	120	96·6	<0·001
3- ,, ...	45	95·7	<0·001
4- ,, ...	14	98·5	<0·001
2 : 3-Dinitrophenol ..	24	98·0	<0·01
2 : 4- ,, ..	3	103·0	<0·01
3 : 5-Dinitro-*o*-cresol ..	3	104·2	<0·02
2 : 5-Dinitrophenol ..	25	95·6	<0·001
2 : 6- ,, ..	6·5	96·8	<0·001
3 : 4- ,, ..	13	99·6	>0·05
3 : 5- ,, ..	7	100·1	>0·10
2-Amino-4-nitrophenol ..	60	96·7	<0·01
4-Amino-2-nitrophenol ..	50	97·1	<0·01

mononitrophenols, 2:3-, 2:5- and 2:6-dinitrophenol, 2-amino-4-nitrophenol and 4-amino-2-nitrophenol all depressed rectal temperature.

Oxygen Consumption.—The effect of the nitrophenols on rate of oxygen consumption was determined with paired rats, one receiving saline and the other the maximum tolerated dose of the nitrophenol. The rats, which weighed 150 to 210 g., were paired for weight and sex; pairs of males and females were used alternately. Decisions were reached by a non-linear Probability Ratio Sequential test (Wald, 1947) of the hypothesis $H_0: |\mu| : < \delta\sigma$ against $H_1: |\mu| : \geqslant \delta\sigma$ where μ is the mean and σ^2 the variance of $\triangle O_2$, the difference in oxygen consumption of the paired rats. δ was chosen as 1, and 0.05 assigned to α and β, the maximum probabilities of errors of the first and second kind. Table IV summarizes the results. The fiducial limits of the mean $\triangle O_2$ establish that 2:4-dinitrophenol and 3:5-dinitro-*o*-cresol stimulated, while 3-nitrophenol, 2:5-dinitrophenol, and both aminonitrophenols depressed, oxygen consumption.

The oxygen consumptions of 120 control rats had an apparently normal distribution, with a mean of 345 ml./hr. and standard deviation 56. A quality control chart confirmed that the variables were in statistical control.

TABLE IV

EFFECT OF NITROPHENOLS ON RATE OF OXYGEN CONSUMPTION OF RATS, DETERMINED BY A NON-LINEAR PROBABILITY RATIO SEQUENTIAL TEST

$\triangle O_2$ = Difference in oxygen consumption of paired treated and control rats. The acceptance of H_0 decides that there is no effect on oxygen consumption, H_1 that there is action on oxygen consumption. The probability of accepting the wrong hypothesis is 0·05.

Compound	Dose (mg.)	No. of Trials for Termination of Test	Hypothesis Accepted	Mean $\triangle O_2$ and 95% Fiducial Limits (ml./hr.)
2-Nitrophenol	105	7	H_0	− 2·9 ± 49·3
4- ,, ...	12	6	H_0	+ 2·0 ± 43·1
2 : 3-Dinitrophenol ..	24	7	H_0	+ 4·0 ± 52·1
2 : 6- ,, ..	6	12	H_0	− 17·0 ± 56·7
3 : 4- ,, ..	13	9	H_0	− 6·0 ± 36·6
3 : 5- ,, ..	6	10	H_0	− 4·8 ± 16·7
3-Nitrophenol	40	10	H_1	− 70·7 ± 49·3
2 : 4-Dinitrophenol ..	3	7	H_1	+ 92·3 ± 51·6
2 : 5- ,, ..	20	21	H_1	− 57·5 ± 36·1
3 : 5-Dinitro-*o*-cresol ..	3	6	H_1	+252·5 ± 39·2
4-Amino-2-nitrophenol	50	6	H_1	− 79·2 ± 40·6
2-Amino-4-nitrophenol	50	11	H_1	− 52·5 ± 35·0

DISCUSSION

In this investigation the inherent variation in biological material was recognized and accepted. No attempt was made to impose "basal" conditions or to enforce total inactivity; variations in feeding, activity, etc., were assumed to be random in accordance with a definite but hypothetical distribution. That the variables were in statistical control was confirmed by satisfactory quality control charts.

Magne *et al.* (1932) classified nitrophenols as metabolic stimulants, metabolic depressants or compounds without action on metabolic rate. This classification may now be regarded as inadequate, since the nitrophenols in this series can be assigned to no less than 6 groups in respect of action on the respiratory exchange of rats (Table V). Nitrophenols with different actions on carbon dioxide output and oxygen uptake were previously unsuspected.

TABLE V

NITROPHENOLS ASSIGNED TO GROUPS FOR ACTION ON RATES OF CO_2 OUTPUT AND OXYGEN CONSUMPTION

Group	Compound	Action on:	
		CO_2 Output	O_2 Uptake
1	2 : 4-Dinitrophenol 3 : 5-Dinitro-*o*-cresol	Stimulant	Stimulant
2	2-Nitrophenol 2 : 3-Dinitrophenol 2 : 6- ,, 3 : 5- ,,	No action	No action
3	3-Nitrophenol 2 : 5-Dinitrophenol	,,	Depressant
4	4-Nitrophenol 3 : 4-Dinitrophenol	Stimulant	No action
5	2-Amino-4-nitrophenol	,,	Depressant
6	4-Amino-2-nitrophenol	Depressant	,,

Of the nitrophenols tested, only those with nitro-groups in both the 2- and 4- positions produce metabolic stimulation in the intact mammal, in spite of former claims that stimulant activity was possessed by 4-nitrophenol, 2:6-dinitrophenol and 3:4-dinitrophenol. The claims for 4-nitrophenol (Gibbs and Reichert, 1894; Magne *et al.*, 1932) and 2:6-dinitrophenol (Tainter and Cutting, 1933), based solely on rectal temperature observations, are weakened by the varying response of this index between species. For example, 2:6-dinitrophenol increases the rectal temperature of pigeons but not of rats (Tainter *et al.*, 1935). Similarly, little significance can be attached to the claim of Magne *et al.* (1932) that 3:4-dinitrophenol increases the rectal temperature and oxygen consumption of dogs, because they admit that the temperature-changes were inconsistent; they measured the oxygen consumption of only one dog treated with 3:4-dinitrophenol.

It appears premature to attempt to correlate the physico-chemical properties and metabolic stimulant activity of the nitrophenols, in spite of previous generalizations by Magne *et al.* (1932) and Clowes and Krahl (1937), since in this series activity is specific for 2:4-dinitrophenol and its methyl derivative, 3:5-dinitro-*o*-cresol.

The stimulation of carbon dioxide output by nitrophenols which are not metabolic stimulants (4-nitrophenol, 3:4-dinitrophenol and 2-amino-4-nitrophenol), although of lesser importance, cannot be ignored. With these nitrophenols, a linear relationship was established between dose and carbon dioxide output, within the dose range examined. Moreover, within the present series, the presence of a nitro-group in the *para*-position to the hydroxyl-group was a necessary and sufficient condition for increasing the carbon dioxide output of rats.

4-Amino-2-nitrophenol and 2-amino-4-nitrophenol were examined as known metabolites of 2:4-dinitrophenol (Guerbet and Mayer, 1932), but their metabolic effects bore no resemblance to those of 2:4-dinitrophenol. Reduction of the nitro-group in the 2-position of 2:4-dinitrophenol has converted a powerful stimulant to a compound which stimulates carbon dioxide output and depresses oxygen consumption; reduction of the nitro-group in the 4-position of 2:4-dinitrophenol has produced a depressant of metabolic rate. The reduction of 2:4-dinitrophenol in the body is therefore a true detoxication.

This investigation has shown that neither rectal temperature nor carbon dioxide output can replace rate of oxygen consumption as a reliable index of metabolic stimulant action. Considerable depression of rectal temperature occurred with compounds having no action on metabolic rate, and it is difficult to avoid concluding that only a temperature increase may have significance. Similarly, the carbon dioxide output of rats can be increased over short periods irrespective of action on metabolic rate, since increased carbon dioxide output was observed with unchanged or depressed oxygen consumption. The use of carbon dioxide output as a simple index of metabolic action, suggested by Benedict and Homans (1911), is therefore not reliable. The apparatus developed in this work measures the oxygen consumption of small mammals simply and accurately, reducing further the importance of carbon dioxide output and rectal temperature as indices of metabolic action.

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ANALGESIC ACTIVITY OF (+)-1-(3-METHYL-4-MORPHOLINO-2 : 2-DIPHENYLBUTYRYL)PYRROLIDINE (R.875) IN STUDENT VOLUNTEERS

BY

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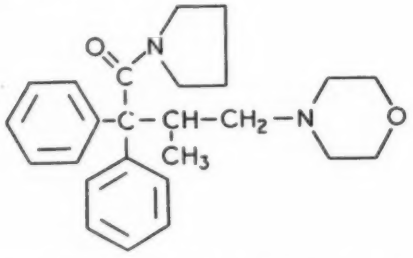
(+)-1-(3-Methyl-4-morpholino-2 : 2-diphenylbutyryl)pyrrolidine (R.875) raised the threshold to ischaemic pain in healthy human volunteers. The peak effect for all doses was reached after about 2 hr. The drug frequently caused nausea and vomiting but no euphoria. Muscular weakness was experienced by many subjects and was sometimes followed 1 to 2 hr. later by hiccup and muscle twitching, suggesting that R.875 may have an effect on striped muscle.

Between 1954 and 1956 Janssen and his co-workers synthesized and examined pharmacologically more than 600 new diphenylpropylamines. Among them was found a number of analgesics, the most potent of which was (+)-1-(3-methyl-4-morpholino - 2:2 - diphenylbutyryl)pyrrolidine. This is an optically active compound with the structural formula—



The racemic form of this compound was designated by Janssen (1956) R.610. The dextrorotatory isomer, which was found in animal experiments to be twice as active an analgesic as the racemic form, was called R.875.

In animal experiments, Janssen and Gageneau (1957), using mechanical and electrical methods of stimulation, found that R.875 was from 8 to 35 times as potent an analgesic as morphine. They also observed that the therapeutic index of R.875 was higher than that of morphine for rats and mice. Janssen (private communication) was unable to demonstrate the development of tolerance to the analgesic effect of R.875 in rats, mice or dogs.

Whilst no estimates are available of the analgesic activity of R.875 in man, a clinical trial of its isomer R.610 reported by Janssen (private communication) indicates that, weight for weight, R.610 is a more potent analgesic than morphine when pain is due to carcinoma, but less effective than morphine in controlling post-operative pain. Janssen found that vomiting rarely occurred in man even after large doses of R.610 or R.875. He reports that there was no visible respiratory depression, foetal or maternal, when subcutaneous doses of up to 40 mg. R.875 were given late in labour. The only side-effect experienced after R.875 was observed after subcutaneous injection of 10 mg. or oral administration of 20 mg. and was a curious feeling of "heaviness in the head" lasting from 15 min. to 1 hr. There was no marked euphoria in the normal subject.

It seemed advisable, as a preliminary to a clinical trial of R.875, to carry out an experiment on human volunteers to find out whether R.875 raises the pain threshold in man, to discover the nature and severity of side-effects, and to construct a dose/response curve for the drug from which doses suitable for a clinical trial could be selected.

METHODS

Selection of Subjects.—21 subjects, who had previously taken part in a similar experiment with dipipanone HCl, were asked to participate in the experiment. This method of selection was used so that a direct comparison between the effects of dipipanone HCl and R.875 on the same subjects could be made. It

also had the advantage that the subjects were already trained in the method to be used.

Doses.—These were selected to form a logarithmic series, namely 2.96, 4.44, 6.67, and 10.0 mg. A dose of 10.0 mg. was chosen as the highest because Janssen has reported that side-effects in man are only observed regularly after doses higher than this. Before beginning the investigation the author received doses of 10 mg. and 20 mg. to ensure that there would be no danger to the subjects from the doses to be used.

All doses were administered in a volume of 1.0 ml. distilled water. The ampoules containing the drug were sterilized before use by autoclaving for 20 min. at 12 to 15 lb. pressure. Distilled water, rather than saline, was used as a solvent because of lack of information regarding the solubility of R.875. As a control, 1.0 ml. distilled water was used. The subjects were unaware that an "inert" control was to be incorporated in the experiment.

Allocation of Doses.—Randomization of doses was not carried out, since this procedure was thought unsafe with a relatively unknown drug. For the same reason a double-blind technique was not employed. The doses were given to each subject in ascending order, but the control injection was given last of all.

Administration of Drugs.—All doses were administered by subcutaneous injection from ampoules which were identical in appearance. The subjects knew the drug only by its code name and were at all times unaware of the doses being used. They were not told anything about the chemical structure of the drug, but they were informed that it was believed to be an analgesic.

Procedure.—The subjects attended the laboratory in small groups on days convenient to them. The only limitation imposed upon their attendance was that at least 48 hr. were allowed to elapse between injections.

The method of estimating pain threshold has been described in detail elsewhere (Cahal, 1957). Pain was produced by ischaemic contractions of the muscles of the forearm, the venous and arterial circulations being occluded by a sphygmomanometer cuff placed around the upper arm and inflated to 220 mm. Hg. The number of ischaemic contractions necessary to produce pain was noted; the subject then continued the contractions until the pain was, in his or her estimation, moderate. The second reading was also recorded and the mean of the two readings taken as an index of pain threshold.

Following a control observation the drug was injected and observations were made at hourly intervals thereafter up to a total of 6 hr. after injection.

After injection the number of contractions necessary to produce pain increased. This increase was regarded as a measure of the rise in pain threshold following injection, and the response was recorded as the difference between the number of contractions necessary to produce pain, as described above, before and after injection.

A space was provided on the record sheet in which subjects could note side-effects.

RESULTS

Two mean observations out of a total of 735 were obtained by extrapolation because on these occasions the subjects were compelled by fatigue to stop contractions before the pain threshold was reached.

Fig. 1 shows that by 4 hr. after injection analgesia had passed its mean peak for the group. The analgesic effect of each dose was therefore

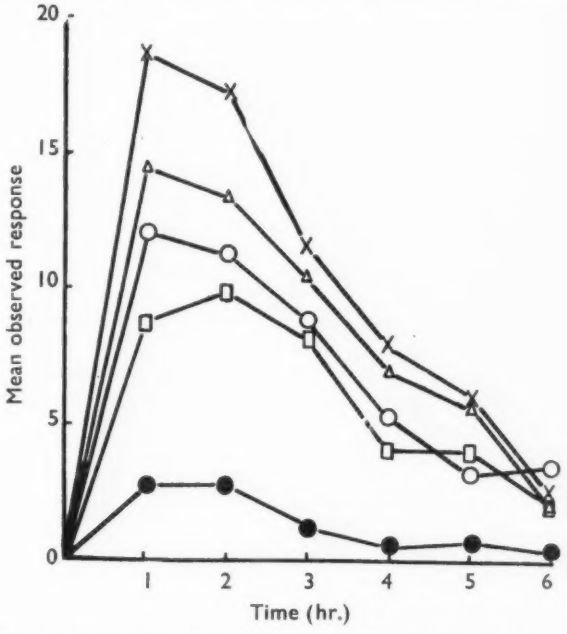


FIG. 1.—Mean observed responses of 21 subjects at hourly intervals after injection of distilled water (●), 2·96 (□), 4·44 (○), 6·67 (△) and 10·0 mg. (×) of R.875. The response is measured as the difference between the number of ischaemic contractions of the forearm muscles necessary to produce pain before and after injection.

estimated by taking the highest individual response for each subject in the first 4 hr. after injection. In this way a few isolated high responses which occurred several hours after injection were excluded.

Table I gives the results of the analysis of variance of the data obtained from the experiments, excluding the control injection, and shows that: (1) There is no evidence that the dose/response relation over the range of doses used is other than linear, (2) there is a significant difference in response both between subjects and between doses, and (3) increasing the dose of R.875 increases the ability of the drug to raise the pain threshold.

TABLE I

ANALYSIS OF VARIANCE OF THE RESULTS OF EXPERIMENTS TO SHOW THE ABILITY OF R.875 TO RAISE THE THRESHOLD TO ISCHAEMIC PAIN IN 21 HUMAN VOLUNTEERS

	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio	P
Regression ..	1,015·26	1	1,015·26	12·85	< 0·001
Linearity ..	279·73	2	139·86	1·77	0·1–0·2
Doses	1,294·99	3	431·66	5·47	0·001–0·01
Subjects ..	3,780·24	20	189·01	2·39	0·01
Error	4,501·76	57	78·98	—	—
Total ..	9,576·99	83	—	—	—

Fig. 2 shows the calculated regression line and the mean observed responses to each dose for the whole group of 21 subjects. The equation expressing the relation between dose and response for this group is also given in Fig. 2.

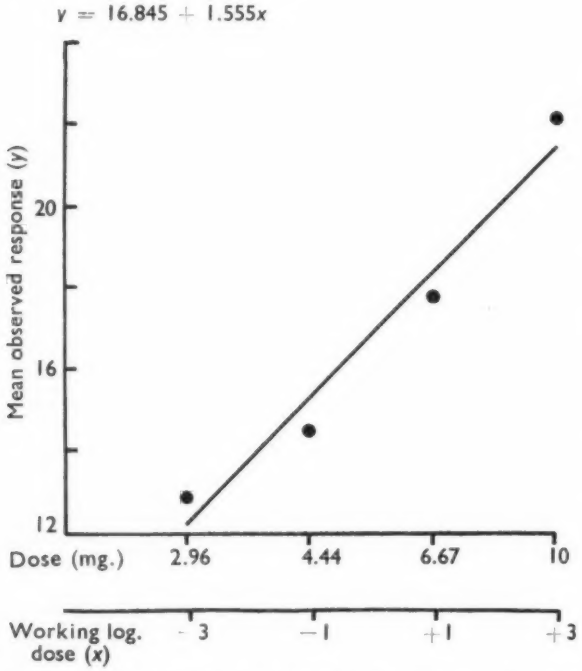


FIG. 2.—Mean observed responses of 21 subjects to R.875 with calculated regression line.

Of the 21 subjects taking part in this experiment, two had been unable to complete a similar experiment with dipipanone hydrochloride because they were hypersensitive to the drug (Cahal, 1957). Therefore, in comparing the effects of R.875 and dipipanone hydrochloride, the results obtained by these two subjects have been excluded.

Table II gives the results of the analysis of variance of the data obtained from the remaining 19 subjects on R.875, and Table III gives the results for the same group on dipipanone hydrochloride. The calculated regression lines with mean observed responses for this group on both dipipanone hydrochloride and R.875 are shown in Fig. 3, which also shows the mean observed responses to the "inert" control injections in both series of experiments.

TABLE II

ANALYSIS OF VARIANCE OF THE RESULTS OF EXPERIMENTS TO SHOW THE ABILITY OF R.875 TO RAISE THE THRESHOLD TO ISCHAEMIC PAIN IN 19 HUMAN VOLUNTEERS

	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio	P
Regression ..	1,441·05	1	1,441·05	19·24	< 0·001
Linearity ..	660·80	2	330·40	4·41	0·1–0·2
Doses ..	880·25	3	293·42	3·91	0·001–0·01
Subjects ..	3,457·00	18	192·06	2·56	0·001–0·01
Error ..	3,819·00	51	74·88	—	—
Total ..	8,156·25	75	—	—	—

TABLE III

ANALYSIS OF VARIANCE OF THE RESULTS OF EXPERIMENTS TO SHOW THE ABILITY OF DIPIPANONE HYDROCHLORIDE TO RAISE THE THRESHOLD TO ISCHAEMIC PAIN IN THE 19 VOLUNTEERS WHOSE RESULTS ARE ANALYSED IN TABLE II

	Sum of Squares	Degrees of Freedom	Mean Square	Variance Ratio	P
Regression ..	3,823·33	1	3,823·33	48·90	< 0·001
Linearity ..	294·92	4	73·73	—	—
Doses ..	4,118·25	5	823·65	10·53	< 0·001
Subjects ..	4,383·12	18	243·51	3·11	< 0·001
Error ..	6,646·25	85	78·19	—	—
Total ..	15,147·62	113	—	—	—

Table IV shows the incidence of side-effects in the group of 21 subjects after distilled water and the four doses of R.875 which were used in these experiments.

DISCUSSION

Fig. 3 shows a striking difference between the mean observed responses to the "inert" control in the experiments on dipipanone hydrochloride and those in the experiments on R.875. The reason is probably that, despite the ignorance of the subjects of the nature of the injection given at any time, the side-effects of R.875 were so marked and so rapid in onset that their absence after an injection of distilled water made it obvious to the subjects that they had had either a very low dose of the drug or an "inert" control injection. This

difference between responses to control injections makes it pointless to attempt any accurate statistical comparison of the pain-threshold-raising capabilities of R.875 and dipipanone hydrochloride. Better experimental design would have overcome this to some extent, because, even though randomization of the doses of R.875 was not considered safe, there was really no reason why, say, half the group should not have had the control injection at the beginning of the series and half at the end. In this way the results of "expectation of side-effects" might have been diminished.

Side-effects of R.875 were very marked even on the lowest dose. They appeared within 5 min. of injection, but rarely lasted for more than an hour. The incidence of nausea even on the lowest dose was almost 50%, and after 10.0 mg. 33.3% of the subjects vomited. It is thus clear from Fig. 3 that, in healthy human subjects, R.875 cannot raise the threshold to ischaemic pain as much as can dipipanone hydrochloride because side-effects limit the dose which can be given. The count of minor side-effects on the higher doses of R.875 is probably inaccurate because many subjects were so distressed by nausea and vomiting that the less unpleasant effects were unnoticed.

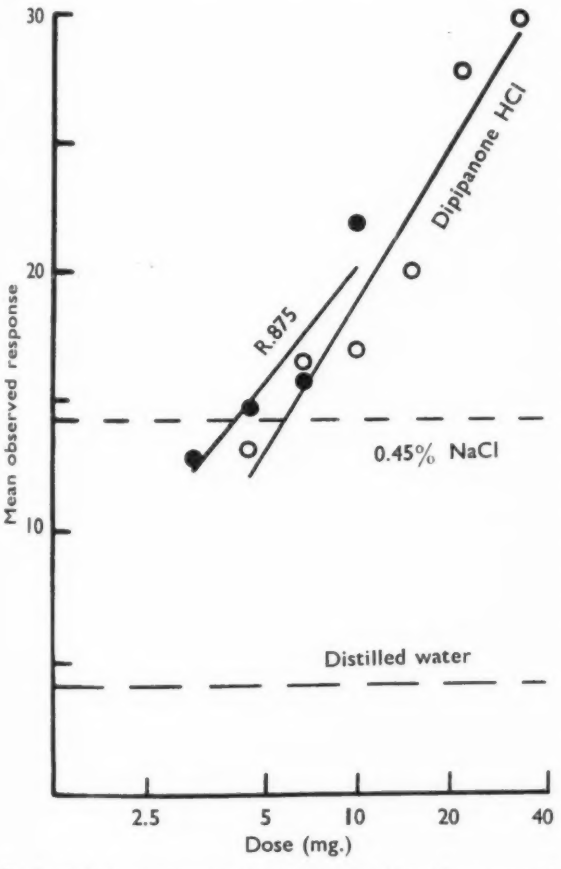


FIG. 3.—Calculated regression lines with mean observed responses of 19 subjects to dipipanone HCl (O) and R.875 (●). The mean observed responses to 0·45% saline and distilled water, which were used as controls in the experiments with dipipanone HCl and R.875, respectively, are shown as broken lines.

TABLE IV

INCIDENCE OF SIDE-EFFECTS AFTER INJECTIONS OF DISTILLED WATER AND VARIOUS DOSES OF R.875

Side-effect	Dose (mg.)					
	Distilled Water	2·96	4·44	6·67	10·0	Totals
Nausea	2	10	10	9	16	47
Vomiting	0	1	3	6	7	17
Dizziness	0	7	10	12	12	41
Drowsiness	1	7	7	10	11	36
Nystagmus	0	4	7	7	12	30
Pruritus	1	3	3	5	9	21
Muscular weakness	0	1	4	5	9	19
Ataxia	0	2	5	4	7	18
Visual disturbance	0	5	3	3	6	17
Headache	2	3	4	5	2	16
Flushing	0	2	2	4	5	13
Dry mouth	0	3	2	3	3	11
Inability to concentrate	0	2	3	1	4	10
Sweating	0	2	2	2	3	9
Shivering	0	0	1	1	4	6
Muzziness	0	3	2	1	0	6
Pallor	0	1	2	2	1	6
Muscle twitches	0	0	0	0	5	5
Hiccup	0	1	0	1	2	4
Sleep	0	0	2	0	2	4
Dissociation	0	1	1	0	1	3
Deafness	0	1	1	0	1	3
Eructation	0	0	0	0	2	2
Inability to swallow	0	0	0	1	1	1
Totals	6	59	74	82	125	346

An interesting observation is the absence of euphoria after the drug. This is most unusual in an analgesic of this potency, and supports the view of Janssen that R.875 produces analgesia without psychical effects.

On 19 occasions volunteers experienced marked muscular weakness, occurring about 30 min. after injection and lasting about half an hour. The frequency of this observation increased with increasing dosage and prompted the suggestion that the drug might have a paralysing effect on striped muscle. Preliminary experiments with the isolated rat diaphragm appear to confirm this, but further work will be necessary before definite conclusions can be reached.

A further indication that R.875 may have an effect on striped muscle is the occurrence of hiccup and muscle twitches. These effects appeared 2 to 3 hr. after injection and may be associated with recovery from paresis of striped muscle.

In conclusion it may be said that R.875 is a potent analgesic. Inspection of the regression lines calculated for the same group of subjects shows that its ability to raise the threshold to ischaemic pain in healthy volunteers is, weight for weight,

equal to or slightly greater than that of dipipanone hydrochloride. The dose which can be given therapeutically is limited by the severity of the side-effects and should probably not exceed 6.0 mg. by subcutaneous injection.

I am indebted to Dr. F. Prescott, of the Wellcome Foundation, for supplies of R.875, to Dr. Paul Janssen for access to his work before its publication, and to the students of the Leeds School of Medicine for their enthusiastic co-operation.

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THE URINARY EXCRETION OF ADRENALINE AND NORADRENALINE BY RATS UNDER VARIOUS EXPERIMENTAL CONDITIONS

BY

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A study has been made of the excretion of adrenaline and noradrenaline in the urine of normal male rats and in the urine of these animals after the subcutaneous injection of saline, adrenaline, noradrenaline, morphine, insulin, iproniazid and thyroxine. The effects of thyroidectomy, adrenal demedullation and ether anaesthesia on the urinary output of adrenaline and noradrenaline have also been investigated. The findings are discussed against the background of the known effects of these treatments on the activity of the sympathetic nervous system.

The results demonstrate that urinary adrenaline and noradrenaline estimations in the rat can, under favourable conditions, serve as an indicator of an alteration of sympathetic activity following the administration of a drug or some other deviation from normal.

In a contemporary paper (Crawford and Law, 1958) we have described a method for the estimation of adrenaline and noradrenaline in urine. The amines are adsorbed on a cation-exchange resin, Amberlite IRC-50, and eluted therefrom with acid. The sympathin (adrenaline + noradrenaline) in the extract may be estimated fluorimetrically under conditions in which the two amines yield equal intensities of fluorescence so that the sympathin estimate is uninfluenced by the proportions of the two amines in the mixture. The two amines may be estimated separately after paper chromatography of the urine extract.

The method has been used to study the urinary sympathin excretion of normal rats and of rats subjected to various experimental procedures. The study was undertaken primarily with a view to demonstrating that measurements of the urinary sympathin excretion would provide a means of detecting alterations in the level of sympathico-adrenal discharge.

METHODS

Adult male albino rats were used throughout.

Urine Collection.—Each rat was housed in a circular galvanized wire gauze cage with a detachable floor consisting of zinc sheet, 21 cm. in diameter, perforated with holes 0.9 cm. in diameter. A hole in the side of the cage just large enough to allow the animal's head to pass through gave access to a compartment into which fitted a dish 6 cm. in diameter for drinking water. The cage was supported by laterally projecting metal rods on top of a large glass filter funnel resting on a rack of angle iron designed to carry six such funnels. The stem of the funnel was cut and bevelled about 3 cm. from the apex. Situated directly underneath the tip of the funnel stem at 2 to 3 cm. distance was the centre of an inverted 100 ml. Kjeldahl flask with the neck reduced to approximately 3 cm. in length. The bulb of the flask rested on three inverted U-shaped pieces of glass rod placed symmetrically round the lip of a glass jar in which the urine eventually collected.

During urine collection, both top and bottom of the floor of the cage was coated with hard paraffin to prevent access of urine to the metal. Faeces usually passed readily through the perforations in the cage floor, down the funnel stem and were deflected off the rounded surface of the inverted Kjeldahl flask. Urine passed down the outer surface of the flask into the collecting jar. Collected in this way, urine samples were reasonably free from faecal contamination.

Drinking water was in continuous supply. Solid food in the form of 15 g. rat cake (North-East Scotland Agricultural Co-operative Society Ltd., Aberdeen) was offered twice daily for 30 min. at 9.15 a.m. and 8.30 p.m. For the feeding, the rat was transferred to a duplicate cage. Before transference, the animal was induced to urinate by gentle pressure on the abdomen in order to prevent urine loss. Such a feeding regimen was found necessary to avoid gross contamination of the urine sample.

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The glass cylinders for urine collection contained 0.5 ml. 2 N-H_2SO_4 (A.R.) to preserve the excreted sympathin (Euler and Hellner, 1951). At the end of the collection, the glass funnel and inverted flask were washed down into the urine specimen with a small quantity of de-ionized water. The 24 hr. specimen from a normal animal was usually about 15 ml., and had a *p*H of 3.0 to 3.5.

Adrenaline and Noradrenaline Estimation.—Estimations of the urinary sympathin (adrenaline+noradrenaline) or of the two amines separately were carried out by the techniques described by Crawford and Law (1958).

For most experiments, six rats were used and usually the 24 hr. urine specimens from pairs of animals were combined. Two portions of 10 ml. were taken from each of two combined samples. One portion was analysed directly to give an estimate of the "free" amines while the second portion was analysed after hydrolysis at *p*H 2 to give an estimate of the "total" (free+conjugated) amines. The other pooled sample was used for estimating the recovery of the amines in the analytical procedure. Factors based on these recovery experiments were used to correct the analytical values (Crawford and Law, 1958).

On occasions, the urine specimens from single animals were analysed either for "free" or for "total" sympathin.

For assays, a fluorimetric method modified (Crawford and Law, 1958) from that of Lund (1949, 1950) was employed routinely. To corroborate the estimates, parallel bioassays were sometimes also performed using the rat blood pressure preparation (Crawford and Outschoorn, 1951), or the isolated rat uterus (Gaddum and Lembeck, 1949).

Administration of Drugs.—Drugs given subcutaneously were injected usually as a neutral aqueous solution in a volume of 0.5 ml. To prevent the loss of fluid during injection, the animal was wrapped in a towel with the head and a portion of the neck exposed and the injection was made into the subcutaneous tissue of the neck. In this way it was possible to perform an injection single-handed without any apparent distress of the animal. However, during the injection the animal frequently urinated, and therefore, when repeated injections were given, the animal was trained to sit quietly on the floor of the cage while the injection was made so that urine voided was not lost.

Control Values for Sympathin Excretion.—The control values for the sympathin excretion for comparison with the excretion after a drug administration were obtained, whenever possible, from previous analyses of the urine from the same rats after the injection of the vehicle in which the drug was to be administered. The injections matched, both in number and in time relations, the proposed injections of the drug solution. Such control experiments were carried out, as far as possible, just prior to those involving the drug.

Results

All estimates recorded are corrected values (see Methods). The values for sympathin, adrenaline and noradrenaline designated "total" refer to estimates obtained from hydrolysed urine; those designated "free" refer to estimates obtained from urine without preliminary hydrolysis; and those designated "conjugated" refer to the difference between the "total" and "free" estimates.

The Effect of Caging of Rats on the Urinary Sympathin Output.—Portions of the pooled 24 hr. urine specimens from three male rats (210 to 230 g.) were analysed. The "total" urinary sympathin output was estimated as 5.0 μg./rat/day during the first day in the metabolism cage, 4.1 μg./rat/day during the fourth day, 1.9 μg./rat/day during the sixth day and 1.9 μg./rat/day during the tenth day. These results indicated an elevated sympathin excretion during the first few days and, consequently, all experiments were carried out only on animals which had been conditioned to the routine of life in the metabolism cages for at least one week.

Daily Excretion of Sympathin by Normal Rats.—Table I gives the estimated daily output of urinary sympathin by normal rats (250 to 260 g.).

TABLE I

URINARY SYMPATHIN EXCRETION OF NORMAL MALE RATS

The rats weighed 250 to 260 g. Fluorimetric and biological assays. The numerals in column (b) refer to the number of animals whose urines were pooled for sampling. The ranges in column (d) indicate the limits between which the estimate was bracketed.

Expt. No. (a)	No. of Rats (b)	Sympathin (μg./rat 24 hr.)	
		Fluorimetric (c)	Rat Blood Pressure (d)
"Total" sympathin			
1	3	2·0	1·1 (1·0–1·2)
2	3	2·4	2·0 (1·5–2·5)
3	1	1·3	1·35 (0·9–1·8)
4	1	2·0	2·1 (1·5–2·7)
	Mean	1·93	1·64
"Free" sympathin			
5	3	1·0	0·9 (0·6–1·1)
6	3	0·9	0·8 (0·6–1·0)
7	3	1·0	0·9 (0·8–1·0)
	Mean	0·97	0·87

Pooled samples were analysed in most cases. Assays of the urine extracts were carried out both fluorimetrically and biologically using (−)-noradrenaline as standard. The estimates by the two methods agreed satisfactorily.

Table II records the estimated daily excretion of adrenaline and of noradrenaline in the urine of normal male rats.

From the combined results cited in Tables I and II, it was estimated that normal rats (250 to 260 g.) excreted 1.15 ± 0.23 (S.D.) (6) μg./day of "free" sympathin, 2.0 ± 0.44 (S.D.) (8) μg./day of "total" sympathin, and 0.67 ± 0.4 (S.D.) (3) μg./day of "conjugated" sympathin. Noradrenaline accounted for 60 to 80% of the "total" sympathin, 60 to 90% of the "free" sympathin and 10 to 100% of the "conjugated" sympathin.

TABLE II

URINARY EXCRETION OF ADRENALINE AND NORADRENALINE BY NORMAL MALE RATS

Samples from individual rats (weighing 250 to 260 g.) were analysed. Fluorimetric assay. The numerals in brackets denote % of noradrenaline or adrenaline in the sum of the two amines

Rat No.	Noradrenaline (μg./rat/24 hr.)		Adrenaline (μg./rat/24 hr.)		Sympathin (μg./rat/24 hr.)		
	"Total"	"Free"	"Total"	"Free"	"Total"	"Free"	"Conjugated"
1	1·19 (62)	1·13 (83)	0·72 (38)	0·23 (17)	1·91	1·36	0·55
2	1·95 (75)	1·3 (88)	0·64 (25)	0·18 (12)	2·59	1·48	1·11
3	1·12 (74)	0·74 (63)	0·4 (26)	0·44 (37)	1·52	1·18	0·34
4	1·86 (79)	Not done	0·48 (21)	Not done	2·34	—	—
Mean	1·53 (73)	1·06 (78)	0·56 (27)	0·28 (22)	2·09	1·34	0·67

Subcutaneous Injection of 0.9% NaCl.—The mean excretion of "free" sympathin after the injection of saline (Table III) showed a significant increase ($P<0.01$) compared with that of untreated rats. The mean amount of "conjugated" sympathin did not show a significant change ($P \sim 0.1$).

Because of these findings the control values for the sympathin excretion for comparison with the excretion after drug administration were obtained, whenever possible, from previous analyses of the urine from the same rats after injection of the vehicle in which the drug was to be administered.

TABLE III

URINARY SYMPATHIN EXCRETION OF MALE RATS IN THE 24 HR. FOLLOWING SUBCUTANEOUS INJECTION OF 0·5 ML. SALINE/RAT

Samples from pooled urine of pairs of rats (each weighing 250 to 260 g.) were analysed. Fluorimetric assay.

Analysis No.	Sympathin (μg./rat/24 hr.)		
	"Total"	"Free"	"Conjugated"
1	4·3	4·9	−0·6
2	4·5	4·2	0·3
3	2·9	2·4	0·5
4	3·2	3·0	0·2
5	2·4	2·5	−0·1
6	2·2	2·0	0·2
7	2·8	2·2	0·6
Mean	3·2	3·03	0·16

Subcutaneous Injection of (−)-Adrenaline or of (−)-Noradrenaline.—Table IV records the excretion of adrenaline and noradrenaline following injection of 1 μg. adrenaline/g. This dose produced visible signs of adrenaline action, such as erection of hair. Table V records the "total" and "free" sympathin excretion following injection of adrenaline (0.1 μg./g.) or of noradrenaline (0.1 μg./g.).

TABLE IV

URINARY EXCRETION OF ADRENALINE AND NORADRENALINE BY MALE RATS IN THE 24 HR. FOLLOWING THE SUBCUTANEOUS INJECTION OF (−)-ADRENALINE (1 μG./G.)

Fluorimetric assays.

Rat No.	(−)-Adrenaline Injected μg.	"Total" Sympathin Excreted as		
		Adrenaline μg.	Noradrenaline μg.	Sum μg.
1	255	20·0	0·55	20·55
2	230	19·7	0·6	20·3
3	240	21·1	1·0	22·1
4	240	23·0	1·1	24·1
Mean	241	20·95	0·81	21·76

Mean sympathin excretion of saline-injected rats (Table III)	3·2 μg.
Mean additional sympathin excretion of adrenaline-injected rats	18·56 μg.
Additional sympathin as % of injected adrenaline	7·7%

TABLE V

URINARY SYMPATHIN EXCRETION BY MALE RATS IN THE 24 HR. FOLLOWING THE SUBCUTANEOUS INJECTION OF (−)-ADRENALINE (0·1 μG./G.) OR OF (−)-NORADRENALINE (0·1 μG./G.)

Samples of pooled urine from pairs of rats (each weighing 265 to 270 g.) were analysed. Fluorimetric assays.

Amine Injected:	Sympathin Excretion (μg./rat)			
	Adrenaline		Noradrenaline	
	"Total"	"Free"	"Total"	"Free"
	6·65	4·05	7·1	7·4
	6·25	4·55	7·6	7·5
	6·3	6·4	7·0	3·15
	7·4	7·55	6·4	4·5
	—	—	6·25	3·9
	—	—	5·2	6·1
	—	—	6·3	6·15
Mean	6·65	5·64	6·55	5·54
Mean saline control excretion	3·2	3·03	3·2	3·03
Additional sympathin excretion after amine injection	3·45	2·61	3·35	2·51
Additional sympathin excretion as % of amine injected	12·9	9·8	12·5	9·4

Subcutaneous Injection of Morphine.—Four injections of morphine hydrochloride (20 μg./g.) were made into each rat at intervals of 1 hr. Urine was collected during the 24 hr. following the first injection. The urine specimens from pairs of rats were pooled for analyses. Control 24 hr.

specimens of urine were obtained the previous day from the same pairs of rats after four subcutaneous injections of 0.5 ml. saline. The results of this experiment (Table VI) showed a definite rise in the urinary sympathin levels after morphine.

An experiment conducted similarly in which the animals received only one injection of morphine hydrochloride (20 μg./g.) gave no real evidence of a change in the sympathin excretion (Table VII).

TABLE VI

URINARY SYMPATHIN EXCRETION OF MALE RATS IN THE 24 HR. FOLLOWING THE FIRST OF FOUR SUBCUTANEOUS INJECTIONS OF MORPHINE HYDROCHLORIDE (20 μG./G.) GIVEN AT HOURLY INTERVALS COMPARED WITH THE EXCRETION OF THE SAME RATS IN THE PREVIOUS 24 HR. FOLLOWING THE FIRST OF FOUR SUBCUTANEOUS INJECTIONS OF 0·5 ML. SALINE/RAT GIVEN AT HOURLY INTERVALS

Samples of pooled urine from pairs of rats (each weighing 265 to 275 g.) were analysed. Fluorimetric assays.

Urine Sample No.	Sympathin Excretion (μg./rat/24 hr.)					
	After Morphine		After Saline		Increase After Morphine	
	"Total"	"Free"	"Total"	"Free"	"Total"	"Free"
1	6·95	5·7	3·4	3·1	3·55	2·6
2	8·6	8·35	4·8	4·7	3·8	3·65

TABLE VII

URINARY SYMPATHIN EXCRETION OF MALE RATS IN THE 24 HR. FOLLOWING SUBCUTANEOUS INJECTION OF MORPHINE HYDROCHLORIDE (20 μG./G.) COMPARED WITH THE EXCRETION BY THE SAME RATS AFTER INJECTION OF 0·5 ML. SALINE/RAT

Samples of pooled urine from pairs of rats (each weighing 260 to 270 g.) were analysed. Fluorimetric assays.

Urine Sample No.	Sympathin Excretion (μg./rat/24 hr.)					
	After Morphine		After Saline		Increase After Morphine	
	"Total"	"Free"	"Total"	"Free"	"Total"	"Free"
1	1·8	2·2	2·2	2·9	−0·4	−0·7
2	4·85	3·4	2·95	3·45	+1·9	−0·05

*Subcutaneous Injection of Iproniazid, 1-*isonicotinyl-2-iso*propyl Hydrazide.*—A study was made of the effect of iproniazid, a monoamine oxidase inhibitor, on the excretion of endogenous and exogenous adrenaline and noradrenaline.

Male rats (265 to 275 g.) were injected subcutaneously with iproniazid [Marsilid phosphate (Roche) 150 μg./g.] and the urine collected during the subsequent 24 hr. Analyses of pooled urine from pairs of rats showed no significant difference in the sympathin excretion/rat from that after a control injection of saline. The excretion of "total" sympathin, estimated in four urine samples, was 2.97 ± 0.64 (S.D.) μg./rat after iproniazid compared with 2.11 ± 0.24 (S.D.) μg./rat after saline in the same rats. The "free" sympathin excretion, estimated using portions of the same urine samples, was 2.08 ± 0.62 (S.D.) μg./rat after iproniazid compared with 2.27 ± 0.3 (S.D.) μg./rat after saline.

The effect of iproniazid on the urinary output of exogenous adrenaline and noradrenaline was also investigated. As controls, three male rats (255 to 270 g). were injected subcutaneously with 0.5 ml. saline followed, 1 hr. later, by an injection of 0.1 μg. (−)-adrenaline/g. or of 0.1 μg. (−)-noradrenaline/g. Urine specimens collected in the 23 hr. following the amine injection were analysed individually for "total" sympathin. One week later the experiment was repeated using the same animals but injecting iproniazid (Marsilid phosphate, 150 μg./g.) in place of the saline. A comparison of the results of the two experiments is shown in Table VIII.

TABLE VIII

EFFECT OF PRIOR ADMINISTRATION OF IPRONIAZID ON THE URINARY SYMPATHIN EXCRETION OF RATS INJECTED (a) WITH 0·1 μG. (−)-ADRENALINE/G.; OR (b) WITH 0·1 μG. (−)-NORADRENALINE/G.

For details see text. Fluorimetric assay. The rats each weighed 255 to 270 g.

(a) Treatment:	"Total" Sympathin Excretion (μg./rat/23 hr.)	
	Saline followed by Adrenaline	Iproniazid followed by Adrenaline
Rat No. 1 ..	4·7	6·4
Rat No. 2 ..	3·45	5·4
Rat No. 3 ..	4·4	5·15
Mean	4·18	5·65
Mean increase in sympathin excretion after iproniazid		1·47 μg.
Mean dose of adrenaline/rat		26·0 μg.
Mean increase in sympathin excretion after iproniazid as % of dose of injected adrenaline		5·7%

(b) Treatment:	"Total" Sympathin Excretion (μg./rat/23 hr.)	
	Saline followed by Noradrenaline	Iproniazid followed by Noradrenaline
Rat No. 4 ..	5·4	7·55
Rat No. 5 ..	4·35	6·2
Rat No. 6 ..	5·25	7·5
Mean	5·0	7·08
Mean increase in sympathin excretion after iproniazid		2·08 μg.
Mean dose of noradrenaline/rat		26·0 μg.
Mean increase in sympathin excretion after iproniazid as % of dose of injected noradrenaline		8·0%

Iproniazid administration appeared to increase the excretion of exogenous adrenaline and noradrenaline. The mean sympathin excretion of the iproniazid-treated rats was found to be just significantly greater in the case of adrenaline and significantly greater in the case of noradrenaline ($P<0.05$) than the mean excretion in the absence of iproniazid.

It must be mentioned, however, that the accuracy of the estimate from the fluorimetric assays of the urine extracts from the iproniazid-treated animals is open to question since some inhibition of the fluorescence derived from adrenaline and noradrenaline added to the extracts was noted. The figures quoted are corrected values based on the estimate of the degree of inhibition noted for each extract which amounted to 30 to 40%.

Subcutaneous Injection of Soluble Insulin.—Male rats were fed at 6 p.m. on the day previous to the experiment. No food was given thereafter until completion of the experiment, but water was in continuous supply throughout. Control sympathin excretion was measured following injection of 0.5 ml. saline/rat. On the following day, soluble insulin *B.P.* (Burroughs Wellcome) 0.5 unit/100 g. was injected subcutaneously. Convulsions, which developed in all the animals within 2.5 to 3 hr., were relieved by the intraperitoneal injection of 20 ml. 6% (w/v) glucose/rat immediately this indication of hypoglycaemia was noticed. The results (Table IX) showed a significant increase in the mean sympathin excretion after insulin compared with that after the control saline injection. The adrenaline and noradrenaline were estimated separately in the urine from rat No. 1 after insulin. The adrenaline fraction amounted to 4.2 μg./rat and the noradrenaline fraction to 2.4 μg./rat. Comparison with the mean values for normal rats indicated that the elevated sympathin excretion after insulin was to be referred mainly to an increase in the adrenaline fraction.

TABLE IX

EFFECT OF SOLUBLE INSULIN, 0·5 UNIT/100 G., SUBCUTANEOUSLY, ON THE "TOTAL" URINARY SYMPATHIN EXCRETION OF FASTING MALE RATS IN THE 24 HR. AFTER INJECTION COMPARED WITH THAT OF A CONTROL SALINE INJECTION

Fluorimetric assay. The rats each weighed 240 to 260 g.

Rat No.	"Total" Sympathin Excretion (μg./rat/24 hr.)	
	After Saline	After Insulin
1	3·15	6·6
2	4·95	6·45
3	3·35	5·25
4	—	6·3
5	—	7·6
6	—	6·8
Mean	3·8	6·5

A similar experiment was carried out on unstarved rats (240 to 265 g.), which each received a subcutaneous injection of soluble insulin, 1 unit/100 g. Both the "total" and the "free" sympathin excretion showed a slight increase over that following a control saline injection, but the mean difference was not significant ($P>0.1$).

Thyroxine.—The sodium salt of L-thyroxine (British Drug Houses) was dissolved in 0.01 N-NaOH to give a concentration of 1 mg./ml. It was administered subcutaneously to six male rats at 10 a.m. and 8 p.m. for three days and at 10 a.m. on the fourth day. The urine excreted in the 24 hr. following the last injection was collected and analysed. A similarly conducted control experiment had previously been carried out on the same rats when each had received a course of injections of 0.01 N-NaOH. The results obtained in these experiments are compared in Table X. No significant difference ($P>0.1$) in the urinary excretion following the thyroxine treatment was detected.

TABLE X

URINARY SYMPATHIN EXCRETION OF MALE RATS FOLLOWING INJECTION OF THYROXINE SUBCUTANEOUSLY (4 DOSES OF 1·0 μG./G.) COMPARED WITH THAT AFTER CONTROL INJECTIONS OF 0·01 N-NaOH

Samples of pooled urine from pairs of rats (each weighing 265 to 275 g.) were analysed. Fluorimetric assay.

Urine Sample No.	Sympathin Excretion (μg./rat/24 hr.)			
	After 0·01 N-NaOH		After Thyroxine	
	"Total"	"Free"	"Total"	"Free"
1	3·4	4·2	4·05	3·75
2	2·8	2·5	3·0	2·65
3	3·45	3·1	4·4	3·45
Mean	3·2	3·25	3·8	3·3

Urinary Sympathin Excretion by Thyroidectomized Rats.—The urinary sympathin excretion of six male rats (300 to 355 g.), following thyroidectomy under ether anaesthesia seven days previously, was compared with that of six rats in the same weight range which had undergone a sham operation at the same time. Three specimens of 24 hr. urine pooled from pairs of rats from each group were analysed for "total" sympathin. In the thyroidectomized group, the sympathin excretion was 2.97 ± 0.1 (S.D.) μg./rat/24 hr., while in the sham-operated group it was 2.78 ± 0.25 (S.D.) μg./rat/24 hr. The difference was not significant ($P>0.1$).

The two groups of rats, thyroidectomized and sham-operated, were treated with thyroxine as in the experiment with normal animals. The effect on the sympathin excretion is shown in Table XI, the individual estimates from the thyroxine treated animals being compared with those previously obtained from the analyses of the pooled urine from the same pairs of rats before treatment.

In both the thyroidectomized and the sham-operated animals, the urinary sympathin excretion showed a consistent rise after thyroxine treatment. However, the sympathin excretion by the thyroidectomized animals after thyroxine did not show a

significant difference (P 0.1 to 0.05) from that of the controls. In the case of the sham-operated animals, the difference was significant ($P<0.01$).

TABLE XI

EFFECT OF SUBCUTANEOUS THYROXINE (4 DOSES OF 1·0 μG./G.) ON THE URINARY SYMPATHIN EXCRETION OF THYROIDECTOMIZED AND "SHAM-OPERATED" MALE RATS

Samples of pooled urine from pairs of rats were analysed. Fluorimetric assay.

Urine Sample No.	"Total" Sympathin Excretion (μg./rat/24 hr.)		
	Untreated	After Thyroxine	Difference
Thyroidectomized rats			
1	3·0	3·45	+0·45
2	2·85	3·05	+0·2
3	3·05	3·25	+0·2
Mean	2·97	3·25	+0·28
"Sham-operated" rats			
1	3·05	3·6	+0·55
2	2·65	3·25	+0·6
3	2·6	3·35	+0·75
Mean	2·77	3·4	+0·63

While these results indicate some rise in the sympathin excretion following thyroxine administration to both thyroidectomized and sham-operated animals, this might be more apparent than real since the basal levels of excretion were probably not adequately controlled inasmuch as the control animals did not receive, before urine collection, a course of injections with the solvent, matching that of the later course of thyroxine injections.

Exposure to Ether.—Four normal male rats (325 to 335 g.) were exposed to ether fumes in a closed box fitted with a Perspex lid for 10 min. after the loss of the righting reflex. The urine excreted in the subsequent 24 hr. was collected. The urine specimens from two rats were combined and "total" adrenaline and "total" noradrenaline estimated. The two estimates of the noradrenaline output were 1.17 and 1.05 μg./rat and of the adrenaline output 0.88 and 1.06 μg./rat. Comparison of these figures with the estimates of the excretion by untreated animals (Table II) indicated that ether probably had no effect on the noradrenaline output but caused a significant rise in the adrenaline excretion ($P<0.05$).

Urinary Sympathin Excretion of Demedullated Male Rats.—The adrenal medullae of eight male rats (315 to 325 g.) were removed under ether anaesthesia. After operation, the rats were kept in individual metabolism cages in a warm room at 21° to 26°. Experiments involving these animals were begun six weeks after the operation. Analyses of the urine from these rats showed the absence of detectable amounts of adrenaline even when the extracts were tested on the very sensitive isolated rat uterus preparation (Table XII). The mean noradrenaline excretion showed no significant difference (P 0.05) from that of normal rats (Table II).

TABLE XII

URINARY SYMPATHIN EXCRETION OF DEMEDULLATED RATS

Samples of pooled urine from pairs of rats were analysed.

Urine Sample No.	"Total" Sympathin (μg./rat/24 hr.)	"Total" Noradrenaline (μg./rat/24 hr.)	"Total" Adrenaline (μg./rat/24 hr.)	
	Fluorimetric Assay		Fluorimetric Assay	Rat Uterus Assay
1	1·05	1·25	<0·06	<0·008
2	0·8	0·7	<0·14	<0·009
3	0·95	1·0	<0·19	<0·009
Mean	0·93	0·98		

Urinary Sympathin Excretion of Demedullated Male Rats after Morphine.—The six demedullated rats used in the previous experiment received four subcutaneous injections of morphine hydrochloride, 20 μg./g. at intervals of 1 hr. The urine excreted during the 24 hr. following the first injection was collected. The urine from pairs of animals was combined, the individual animals of each pair being the same as in the previous experiment (Table XII). The "total" adrenaline and the "total" noradrenaline contents of the urine samples were determined. The adrenaline was estimated both fluorimetrically and biologically, using the isolated rat uterus.

The estimated noradrenaline excretion, μg./rat, was 1.56, 1.67 and 1.63 in the three samples analysed, and the mean excretion was just significantly greater ($P<0.05$, >0.02) than that of the untreated demedullated animals. The estimate of the adrenaline excretion was less than 0.1 μg./rat (fluorimetric) and less than 0.002 μg./rat (rat uterus) for all three samples indicating the absence of any detectable adrenaline from the urines (<0.2% of the noradrenaline content).

Exposure of Demedullated Rats to Ether.—Four demedullated rats (315 to 325 g.) were exposed to ether fumes and the urine collected and analysed in the same manner as in the experiment on normal rats. The estimates of the noradrenaline excretion were 0.96 and 0.83 μg./rat/24 hr. They did not indicate any real difference between the excretion by ether-exposed and by untreated demedullated animals (Table XII). Neither urine sample showed the presence of any detectable amounts of adrenaline, the excretion being estimated at <0.05 μg./rat/24 hr. (fluorimetric) and <0.007 μg./rat/24 hr. (rat uterus).

Discussion

The urinary sympathin (adrenaline and noradrenaline) excretion of male rats under various experimental conditions has been studied using the analytical techniques described by Crawford and Law (1958), who have discussed the accuracy and specificity of these methods

In the following discussion of the results, estimates classified as " total " refer to the sympathin which is detectable after hydrolysis of the urine at *p*H 1.8 to 2.0 and which includes the " free " sympathin, detectable without hydrolysis, and some, but not all, of the " conjugated " sympathin (see Crawford and Law, 1958, for discussion and references).

Normal Rats.—For the first few days in the individual metabolism cages the animals were restless and disinclined to eat. After 5 to 6 days they appeared to have become used to their new environment. The initial restlessness was paralleled by a relatively high " total " sympathin excretion which subsequently fell to a more or less steady level. This observation pointed to the necessity for using conditioned rats for the study of the effect of drugs and other deviations from the normal on the sympathin excretion. The animals were therefore kept in the metabolism cages for at least one week before use.

Male rats thus conditioned and weighing about 250 g. had a " total " sympathin output of about 2.0 μg./day of which some 50 to 70% was " free." Noradrenaline was the predominant amine in the " free " sympathin (60 to 90%) but not always in the " conjugated " sympathin (10 to 100%). Pitkänen (1956) found the " free " sympathin excretion by rats over a 5 hr. collection period to be 0.36 ± 0.16 (S.D.) μg. of which 14 to 80% was noradrenaline.

The main source of adrenaline in normal rat urine would appear to be the adrenal medulla, since none was detectable in the urine of demedullated rats. The urinary noradrenaline levels of such animals did not differ significantly from that of normal rats, indicating that this amine was derived mainly, if not entirely, from extramedullary sources. Similar conclusions for men were drawn from studies of the adrenaline and noradrenaline excretions of adrenalectomized (Euler, Franksson and Hellström, 1954; Elmadjian, Lamson, and Neri, 1956) and sympathectomized subjects (Goldenberg and Rapport, 1951). Pitkänen (1956) found that the mean adrenaline excretion of adrenalectomized rats receiving cortisone acetate was about one-third of the mean normal excretion, but the amine was still present in detectable amounts in all urine specimens analysed.

Saline.—The subcutaneous injection of saline increased the excretion of " free " sympathin. This observation indicated that it would be more accurate to compare the sympathin excretion following a drug injection not with that of the normal untreated animal but with that of the same animal after the injection of whatever vehicle was to be used in administering the drug.

Adrenaline and Noradrenaline.—After the subcutaneous injection of (−)-adrenaline or (−)-noradrenaline the 24 hr. " total " sympathin excretion was increased by an amount corresponding to about 10% of the injected dose irrespective of the amine. The experimental results for both amines indicated that the additional sympathin might be wholly or only partly in the " free " form. The injection of adrenaline did not affect the normal level of noradrenaline excretion. These results agree with previous findings that only a small fraction of injected adrenaline or noradrenaline can be accounted for by urinary excretion of the amines in the rat (Schayer, 1951a and b), in the rabbit (Fischer and Lecomte, 1950), in the dog (Bacq, Fischer, Lecomte, and Verly, 1951) and in man (Euler and Luft, 1951; Goldenberg, 1951; Euler, Luft, and Sundin, 1954; Euler and Zetterström, 1955; Pekkarinen and Pitkänen, 1955; Elmadjian *et al.*, 1956). It is inferred therefrom that the main bulk must suffer enzymatic destruction in the body, and it has been postulated that monoamine oxidase is an important enzyme in this connexion (Blaschko, 1952; Burn, 1951, 1952). It might be expected, therefore, that the administration of a monoamine oxidase inhibitor to an animal would result in an increased urinary sympathin excretion. One substance highly active as an amine oxidase inhibitor is iproniazid, 1-*iso*nicotinyl-2-*iso*propyl hydrazide (Zeller, Barsky, Fouts, Kirchheimer and Orden, 1952; Zeller and Barsky, 1952).

Iproniazid.—The subcutaneous injection of iproniazid produced no detectable change in the urinary excretion of endogenous sympathin in the 24 hr. following the injection. The excretions of exogenous adrenaline and noradrenaline showed significant increases over the control levels when the amines were injected 1 hr. after iproniazid. The increases were, however, not marked. These observations would indicate that monoamine oxidase plays a minor rôle in the metabolism of the catecholamines. A similar conclusion may be drawn from the observation of Euler and Zetterström (1955) that, in man, subcu-

taneously injected cobefrine (1-(3′,4′-dihydroxyphenyl)-2-aminopropanol), a catecholamine resistant to monoamine oxidase was inactivated in the body to about the same extent as subcutaneously injected adrenaline and noradrenaline. On the other hand, Schayer and his co-workers (Schayer, Smiley, and Kaplan, 1952; Schayer, Smiley, and Kennedy, 1953; Schayer, Wu, Smiley, and Kobayashi, 1954) obtained evidence that, in the rat at least, monoamine oxidase destroyed about half of a dose of injected adrenaline while most of the remainder was metabolized by some other enzyme system. In the presence of a monoamine oxidase inhibitor (iproniazid or choline-*p*-tolyl ether), this other enzyme system took over almost the entire metabolism. Such being so, monoamine oxidase inhibition would not be expected to lead to any marked increase in the urinary excretion of injected adrenaline (or noradrenaline), a conclusion consistent with the results of the present study.

Morphine.—The administration of morphine to animals leads to the release of adrenal-medullary hormones, principally adrenaline (Elliott, 1912; Stewart and Rogoff, 1916, 1922; Outschoorn, 1952; Vogt, 1954). In the present study, a single dose of morphine hydrochloride (20 μg./g.) produced no significant rise in the sympathin excretion in the subsequent 24 hr. urine. A significant rise, mainly related to the "free" sympathin, followed administration of this same dose of morphine four times at intervals of 1 hr. Demedullated rats treated similarly did not show such a marked rise in sympathin excretion, pointing to the adrenal medulla as the main source of the excess sympathin in the urine of normal rats treated with morphine. These findings are consistent with the observations of Outschoorn (1952), who detected no significant change in the catecholamine content of rat adrenal glands after a single injection of morphine hydrochloride (20 μg./g.) but a considerable depletion after four such doses given at intervals of 1 hr.

The urinary excretion of noradrenaline by demedullated rats was slightly increased after morphine, indicating a general sympathetic stimulation by the drug rather than an action limited to the adrenal medulla. This is consistent with past observations (Elliott, 1912; Vogt, 1954).

Ether.—The excretion of noradrenaline following exposure of normal rats to ether did not differ significantly from that of untreated animals, but there was an indication of an elevated adrenaline excretion. No adrenaline was detected in the urine of demedullated rats treated with ether, and the noradrenaline output of such animals showed no significant alteration from that of untreated demedullated animals. The changes in urinary sympathin were thus found to reflect the changes in plasma adrenaline levels found by Vogt (1952b) to occur under essentially similar experimental conditions.

Insulin.—Insulin causes a depletion of adrenal medullary hormones, principally adrenaline (Vogt, 1947; Burn, Hutcheon, and Parker, 1950; Hökfelt, 1951; West, 1951; Outschoorn, 1952; Udenfriend, Cooper, Clark, and Baer, 1953). A rise in the plasma adrenaline concentration following insulin administration has been demonstrated in the fasting man and dog by Holzbauer and Vogt (1954), in man by Millar (1956), and in the cat by Dunér (1954). In the present work, soluble insulin injected subcutaneously into fasting rats produced a two- to three-fold increase in the "total" sympathin excretion. Evidence from one experiment indicated that the increase was mainly of adrenaline. The administration of insulin to unstarved animals produced a small but not significant ($P>0.1$) rise in the sympathin excretion. Pitkänen (1956) found up to a twenty-fold increase over the basal adrenaline excretion of fasting rats in the 5 hr. following insulin, while Euler and Luft (1952) have reported increases of up to tenfold in the adrenaline excretion of man after insulin.

Thyroxine and Thyroidectomy.—Neither thyroxine administration nor thyroidectomy produced significant alteration in the urinary sympathin excretion of male rats. Thyroxine did, however, raise the output of "total" sympathin in both thyroidectomized and "sham-operated" animals, the increase being almost significant ($P<0.1$, >0.05) in the case of the thyroidectomized animals and significant ($P<0.01$) in the case of the "sham-operated" animals.

Spinks and Burn (1952) detected a small decrease in the monoamine oxidase content of the liver following thyroid feeding in rabbits, while thyroidectomy produced a small effect in the opposite direction in rabbits and rats. Trendelenburg (1953) confirmed the earlier findings of Burn and Marks (1925) that thyroid feeding increased adrenaline hyperglycaemia in rabbits, an effect which he suggested might be due to a decrease in the monoamine oxidase content of the liver.

In view of the absence of a marked effect on the sympathin excretion of rats after iproniazid, it seems unlikely that the slight increase noted after thyroxine was due to alteration in the monoamine oxidase levels in the animals. In this connexion it may be remarked that Schayer (1953)

has found that tyramine metabolism in mice was significantly altered by amine oxidase inhibitors such as iproniazid but not by thyroxine, a finding which pointed to the absence of any marked alteration of the monoamine oxidase levels following administration of the latter drug.

The results of the various experiments reported in this paper indicate that the estimation of the sympathin excretion in the urine of rats can, under favourable conditions, serve to demonstrate alterations in the normal level of circulating adrenaline or noradrenaline. Its usefulness appears, however, to be somewhat restricted since it is likely that only relatively large changes will be detected. Only about 10% of injected adrenaline or noradrenaline is excreted in the urine in a form which can be estimated by the method. On the assumption that a similar fraction of the endogenous amines is excreted, a mean output of about 2 μg./day represents the release into the blood stream of the animal of some 20 μg. of sympathin, or about 1 μg./hr. The additional release of, say, 5 μg. of sympathin during the hour following a drug injection would result in an increase of only 0.5 μg. in the sympathin in the urine sample collected during the 24 hr. after the injection although this additional quantity would be excreted within a much shorter period. Owing to the variability of the normal 24 hr. urinary sympathin excretion (S.D. about 0.5 μg.), a mean rise of this order obtained in an experiment involving only two or three animals would probably not be significant. It seems probable that this argument affords an explanation of the failure in some of the experiments to demonstrate definitely an expected stimulation of the sympathetic system as, for example, during ether anaesthesia. This difficulty could be overcome to some extent at least by decreasing the interval of urine collection after the drug administration and thus decreasing the normal background level of the sympathin so that any additional excretion would be more likely to reach a statistically significant level. This possible modification of the technique has not been investigated by us, but Pitkänen (1956) has reported experiments on urinary sympathin estimations in rats in which the excretion was collected over periods as short as 2 hr., the urine output being increased by prior administration of water by stomach tube.

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THE RENAL REMOVAL OF INJECTED [^{14}C]HISTAMINE FROM THE BLOOD IN DOGS

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The amounts of [^{14}C]histamine in arterial blood, in renal venous blood and in urine during a steady intravenous infusion of [^{14}C]histamine were measured in five anaesthetized dogs. The extraction ratio for [^{14}C]histamine was 0.7 to 0.8, indicating that the histamine was efficiently taken up from the blood by the kidney. Only a small part (14 to 18%) of the [^{14}C]histamine removed from the blood by the kidney appeared in the urine as histamine. Evidence was obtained that [^{14}C]histamine was metabolized in the kidney not only by histaminase (diamine oxidase) but also by the histamine methylating enzyme.

The capacity of the canine kidney to inactivate histamine is well known since the investigations of Best and McHenry (1930). Some evidence has been obtained that only part of this capacity is due to the histaminase (diamine oxidase) of the kidney (Lindell, 1957a and b). The use of [^{14}C]histamine offered the possibility of studying the renal removal of injected histamine at blood concentrations of histamine sufficiently low to have little or no observable effects on the blood pressure. In the present study [^{14}C]histamine was given intravenously at a slow, steady rate and the amounts of [^{14}C]histamine in arterial blood, in renal venous blood and in urine were measured. The rôle of histamine-metabolizing enzymes in the renal removal of injected histamine was estimated in two ways. Firstly, from the effects of a potent histaminase inhibitor, aminoguanidine, on the renal extraction and excretion of [^{14}C]histamine. Secondly, from measurements of the amounts of known metabolites of [^{14}C]histamine in arterial blood, in renal venous blood and in urine.

METHODS

The dogs, which had been fasted overnight, were anaesthetized with pentobarbitone sodium as described by Lindell (1957a). The region of the renal vessels was exposed through an incision in the right hypochondrium. A polythene tube (PE 240, Clay Adams Comp. Inc., New York) with a suitably bent tip was inserted into a renal vein from a femoral vein under direct vision. If the left renal vein was used the ovarian vein was ligated. Polythene tubes (PE 100) were inserted into the ureters through the same incision on the right side and through a lower abdominal incision on the left side. Arterial blood was collected from a polythene tube in a femoral artery. Polythene tubes for the administration of pentobarbitone sodium, [^{14}C]histamine, *p*-aminohippuric acid (PAH), dextran [Macrodex (Pharmacia)], saline and aminoguanidine were introduced into suitable veins in the legs. The rectal temperature was maintained at approximately 38° as in the previous experiments (Lindell, 1957a). All injections were made with motor-driven syringes. During the operative procedure 50 to 100 ml. of 0.6% saline was given intravenously to increase the diuresis. The [^{14}C]histamine was dissolved in saline to which glucose was added (approximately 1 mg./ml.). The glucose helps to stabilize the histamine solution (Schayer, unpublished observation). The infusion of [^{14}C]histamine was at the rate of 0.3 to 0.9 μg./kg./min. and had little or no effect on the blood pressure recorded with a mercury manometer from the carotid artery. The [^{14}C]histamine was given for 20 to 30 min. before the start of the collection of urine. The urine collection periods were 15 to 40 min. and the volumes of urine collected in these periods were 2 to 10 ml. The arterial and renal venous blood samples were taken simultaneously. The blood flowed spontaneously from the catheter in the renal vein. The dogs were given 5 mg. heparin every hour. Blood losses were compensated for by a steady infusion of dextran. At the end of the infusion of histamine the kidneys were removed and weighed and in some experiments extracted for [^{14}C]histamine as described below.

Assay for [^{14}C]Histamine and its Metabolic Products in Blood.—The blood, usually 5 ml. for each sample, was taken into glass tubes containing 0.5 mg. heparin. The carrier [histamine, methylhistamine {4-(2-aminoethyl)-1-methylimidazole}, methylimidazoleacetic acid (1-methylimidazol-4-ylacetic acid) or imidazoleacetic

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acid] was added immediately. The sample was then thoroughly mixed and allowed to stand for at least 30 min.; 20% trichloroacetic acid (TCA) was added to give a final concentration of about 5%. After mixing, the sample was allowed to stand overnight. It was then filtered and the precipitate was washed repeatedly with 5% TCA. TCA was removed with ether before further extraction took place. The further treatment of the various samples was as follows:

[^{14}C]Histamine.—The histamine was extracted with butanol (Schayer, 1952). The dipicrate was prepared, converted to pipsylhistamine (Schayer and Cooper, 1956) and counted.

Methylhistamine.—This was extracted with chloroform (Rotschild and Schayer, unpublished observation) and the picrate prepared and counted.

Methylimidazoleacetic Acid.—After removal of TCA, the filtrate was passed through a Dowex 50 column in the acid form. The methylimidazoleacetic acid was eluted with ammonia solution. The volume of the eluate was reduced by evaporation and the picrate prepared and counted (Schayer and Cooper, 1956).

Free Imidazoleacetic Acid.—The picrate was prepared in the same way as with methylimidazoleacetic acid. It was then converted to imidazoleacetic acid hydrochloride by passage through a Dowex 1 column. Pipsylimidazoleacetic acid was prepared and counted.

Imidazoleacetic Acid Riboside.—This compound was estimated as the difference between total imidazoleacetic acid (the value obtained after hydrolysis as described by Schayer and Cooper, 1956) and free imidazoleacetic acid.

Determination of [^{14}C]Histamine and its Metabolites in Urine.—The urine was collected in glass tubes containing 0.5 ml. 6 N-HCl. The volume of urine used for each assay was usually 0.5 ml. to which carrier was added and the respective picrate prepared directly. The picrates were then treated as described above.

Estimation of the Amount of [^{14}C]Histamine in the Kidney at the End of the Infusion of [^{14}C]Histamine.—The kidneys from the dogs which had been given aminoguanidine were removed at the end of the infusion of histamine and packed in ice to obtain rapid cooling. They were then stored at −15°. At an early stage of thawing the renal tissue was cut with scissors, an aliquot was then put in a homogenizer together with 0.01 N-HCl and carrier added. After homogenization, TCA was added and the samples treated in the same way as the blood samples.

The samples were counted at infinite thickness in a flow counter, the background of which was 19 to 22 c./min. The counts of the blood samples ranged from 6 to 150 above background. The samples were recrystallized until constant radioactivity. At least 1,000 counts were taken after each recrystallization.

The concentrations of [^{14}C]histamine or metabolites in whole blood in the results are given in counts/min. above background/ml.(c./min./ml.). The concentration of [^{14}C] in histamine in whole blood when given in c./min./ml. may be converted to μg. histamine base/l. blood by multiplication by 0.5. The amounts of [^{14}C]histamine or metabolites in the urine are given in counts/min. above background (c./min.) excreted/min. To make the values for histamine and metabolites comparable, the counts obtained for pipsylhistamine and pipsylimidazoleacetic acid have been recalculated to the corresponding values in the form of picrates.

Measurement of Renal Blood Flow.—In two experiments the renal blood flow was estimated from the extraction and excretion of PAH with the use of the Fick principle. PAH (10 mg./kg.) was given as a priming dose which was followed by a continuous intravenous infusion of PAH at a rate of 5 to 6 mg./min. Arterial and renal venous blood samples for the determination of PAH were taken in the same way as the samples for assay of [^{14}C]histamine and its metabolites. The urine for PAH analysis was an aliquot from the urine sample used for assay of [^{14}C]histamine. The colorimetric determinations of PAH in whole blood and in urine were carried out at the Department of Clinical Physiology, Karolinska, Sjukhuset, Stockholm. In preliminary experiments, it was found that 85 to 90% of PAH added to whole blood was recovered. In the calculation of renal blood flow no correction has been made for this 10 to 15% loss.

The [^{14}C]histamine was obtained from the Radiochemical Centre, Amersham, England. The aminoguanidine was in the form of the sulphate (Eastman Organic Chemicals, Rochester, U.S.A.).

RESULTS

The first experiment was designed to show the renal extraction and excretion of [^{14}C]histamine during a steady intravenous infusion of [^{14}C]histamine (0.4 μg./kg./min.). The design and the results are summarized in Table I. Two arterial blood samples and two renal venous blood samples were taken in the middle of each urine collection period. Urine was collected in five periods spaced over nearly 4 hr. Three of these collections, I, III and V, took place during intravenous infusion of [^{14}C]histamine. The urine collections of periods II and IV took place 50 min. after interruption of the histamine infusions There was a good agreement between the duplicates in the assay of [^{14}C]histamine in blood. The extraction ratio ($\frac{A-V}{A}$, where A is the concentration of [^{14}C]histamine in arterial whole blood and V the concentration in renal venous blood) was 0.7 to 0.8 in periods I, III and V. The amount of [^{14}C]histamine excreted in these periods was nearly

TABLE I

Female dog, 9 kg. Catheter in right renal vein for collection of renal venous blood. Arterial blood taken from the right femoral artery. Urine collected from a catheter in the right ureter. Weight of the right kidney 24 g. The blood pressure was between 110 and 130 mm. Hg. during the experiment. The concentration of [^{14}C]histamine in whole blood is given in c./min./ml., which may be converted to μg. histamine base/l. blood by multiplication by 0·5. The counts above background obtained for the blood samples were about 5 times greater than those given in the Table, since the volumes of the samples were about 5 ml.

Time in Min.	Intravenous Infusion of [^{14}C]Histamine 0·4 μg./kg./min.	Period No.	Collection of Urine	Sampling of Blood	Conc. of [^{14}C]Histamine in Whole Blood (c./min./ml.)		Extraction Ratio	Amounts of [^{14}C]Histamine Excreted by the Kidney/min. (c./min.)
					Arterial	Renal Venous		
0								
20					5·1	1·1	0·8	
		I		←	5·5	1·6	0·7	72
40								
60								
80								
100		II		←	0·7	—	—	0
120				←	6·6	1·7	0·7	
		III		←	6·2	Lost	—	70
140								
160								
180								
		IV						1
200								
220				←	5·0	1·6	0·7	
		V		←	5·4	1·8	0·7	68
240								

the same, 68 to 72 c./min. being excreted/min. by the right kidney. Fifty minutes after the cessation of the infusion of [^{14}C]histamine, the urinary excretion of [^{14}C]histamine was 1 c./min. or less/min. The clearance value for [^{14}C]histamine calculated in the conventional way was about 0.5 ml. whole blood/min./g. of kidney in periods I, III and V.

The Effect of Aminoguanidine on the Renal Removal of [^{14}C]*Histamine.*—The results of the second experiment, which have been summarized in Table II, had in principle the same design as the first one. However, between periods III and IV aminoguanidine was given intravenously in a dose of 12 mg./kg. During periods I and II (Table II), the extraction ratio for [^{14}C]histamine was 0.7 to 0.8, being the same as in the preceding experiment. The amount of [^{14}C]histamine excreted/min. by the right kidney was 140 to 180 c./min. The clearance value was thus 0.6 to 0.7 ml. whole blood/min./g. of kidney. In period III 50 min. after the cessation of the infusion of [^{14}C]hist-

TABLE II

Female dog, 10 kg. The same experimental conditions obtained as in Table I. The blood pressure was 120 to 150 mm. Hg. Weight of right kidney 29 g.

Time in Min.	Intravenous Infusion of [^{14}C]Histamine 0·45 μg./kg./min.	Period No.	Collection of Urine	Sampling of Blood	Conc. of [^{14}C]Histamine in Whole Blood (c./min./ml.)		Extraction Ratio	Amounts of [^{14}C]Histamine Excreted by the Kidney/min. (c./min.)
					Arterial	Renal Venous		
0	⌐							
20	¦	I	⌐	←	7·0	1·8	0·7	140
40	¦	II	¦	←	10	2·2	0·8	180
60	⌙		⌙					
80								
100		III	⌐					20
120	⌐		¦	Aminoguanidine 12 mg. kg. (i.v.)				
140	¦	IV	⌐	←	10	5·7	0·4	370
160	¦	V	¦	←	12	7·0	0·4	390
180	⌙		⌙					

amine, the amount excreted by the kidney/min. was 20 c./min. After the administration of aminoguanidine (periods IV and V), the extraction ratio fell to 0.4, while the amount of [^{14}C]histamine excreted by the kidney/min. increased to 370 and 390 c./min. The corresponding clearance values were 1.3 and 1.1 ml. whole blood/min./g. of kidney. The right kidney removed at the end of the infusion was extracted for [^{14}C]-histamine. The amount of [^{14}C]histamine found in the kidney was about 600 c./min.

To calculate the amount of [^{14}C]histamine removed from the blood by the kidney it was necessary to measure the blood flow through the kidney simultaneously with the estimations of [^{14}C]histamine in arterial and renal venous blood. In the third experiment in this series, PAH was given in a continuous intravenous infusion and the renal extraction and excretion of PAH were measured. From the values obtained, the blood flow through the kidney was calculated according to the Fick principle. The figures for renal blood flow, calculated in this way, were 150 to 210 ml./min. The results of this experiment have been summarized in Table III. It may be seen in the Table that the extraction ratio for [^{14}C]histamine in period I is 0.7 or the same as found in the two preceding experiments before the administration of aminoguanidine. After the injection of the histaminase inhibitor, the extraction ratio fell to 0.4 to 0.5 as in the previous experiment. The amount of [^{14}C]histamine removed by the kidney from the blood was calculated from the arterio-venous difference in the concentration of [^{14}C]-histamine and the figure for renal blood flow obtained in the PAH studies. In period I, approximately 14% of the [^{14}C]histamine removed from the blood by the kidney was excreted in the urine. After aminoguanidine, this % increased to 25 to 40. The clearance values, calculated in the conventional way, were, in period I, 0.6; in period II, 0.9; in period V, 0.5; and in period VI, 0.6 ml. whole blood/min./g. of kidney. The amount of [^{14}C]histamine found in the kidney removed at the end of period VI was 2,000 c./min. which may be compared with the 1,800 c./min. removed from the blood by the kidney/min. during the last urine collection period.

TABLE III

Female dog, 10 kg. Renal venous blood and urine were collected from the left kidney, otherwise the experimental conditions were as described in Table I. The blood pressure was 120 to 155 mm. Hg. Weight of left kidney 36 g.

Time in Min.	Intravenous Infusion PAH 10 mg./kg.	Period No.	Collection of Urine	Sampling of Blood	Conc. in Whole Blood of PAH (mg./100 ml.) [^{14}C]Histamine (c. min./ml.)		Amount of PAH Excreted by Kidney (mg./min.)	Blood Flow through the Kidney (ml./min.)	Amount of [^{14}C]Histamine Removed by the Kidney min. (c. min.)	Amount of [^{14}C]Histamine Excreted by the Kidney min. (c. min.)
					Arterial Blood	Renal Venous Blood				
0	PAH 6 mg. min.									
20		I		PAH ←[^{14}C]Histamine	2·40 8·4	1·20 2·4	2·54	210	1,300	180
40	[^{14}C]Histamine 0·5 µg./min.									
60										
80		II								8
100					Intravenous injection of aminoguanidine 12 mg. kg.					
120		III		PAH ←[^{14}C]Histamine	3·0 13	1·20 6·0	2·63	150	1,100	410
140	[^{14}C]Histamine 0·5 µg./min.									
160										
180		IV								33
200										
220	[^{14}C]Histamine 0·3 µg./min.	V		PAH ←[^{14}C]Histamine	3·20 9·6	2·00 6·1	2·52	210	740	190
240										
260	[^{14}C]Histamine 0·9 µg./min.	VI		PAH ←[^{14}C]Histamine	4·95 22	3·20 11	2·86	160	1,800	450
280										

The results obtained so far indicate: (1) That injected histamine may be relatively efficiently removed from the blood by the canine kidney, the extraction ratio being 0.7 to 0.8 (whole blood). (2) That only a small part (14%) of this removed histamine appears in the urine. (3) That the histaminase of the kidney is partly responsible for the renal removal of histamine, the evidence being that the extraction ratio decreased to 0.4 to 0.5 after the administration of a histaminase inhibitor. (4) That even after the injection of the inhibitor more histamine may be removed from the blood by the kidney than is excreted in the urine.

Since there was no evidence for the storage of any considerable amount of [^{14}C]histamine in the kidney, it seems not unlikely that some of the renal removal of histamine persisting after the administration of a histamine inhibitor is due to the action of a histamine metabolizing enzyme different from histaminase. This may be the methylating enzyme (Schayer and Karjala, 1956), which leads to the formation of methylhistamine

TABLE IV

Female dog, 13 kg. Urine and blood collected as described in Table I, but from the left kidney, which weighed 41 g. The blood pressure was 110 to 140 mm. Hg. The excretion of PAH was 2·33 mg./min. and the blood flow through the left kidney was 194 ml./min. (calculated according to the Fick principle). The amount of [^{14}C]histamine or its metabolites removed from the blood by the kidney/min. was calculated from the arterio-venous differences in the concentrations of these substances and from the figure for the blood flow through the kidney.

Time in Min.	Intravenous Infusions	Collection of Urine	Sampling of Blood	Conc. in Whole Blood PAH (mg./100 ml.) [^{14}C]Histamine and its Metabolites (c./min./ml.)		Amount of [^{14}C]Histamine or Metabolite Removed from the Blood by the Kidney/min. (c./min.)	Amount of [^{14}C]Histamine or Metabolite Excreted by the Kidney/min. (c. min.)
				Arterial	Renal Venous		
0	PAH 5 mg./min.						
30	[^{14}C]Histamine 0·5 /g. kg./min.						
36			PAH	1·75	0·55		
			Histamine	15	4·4		
			Methylhistamine	8·2	3·8		
			Methylimidazoleacetic acid	21	16		
			Total imidazoleacetic acid	13	Lost		
			PAH	1·80	0·60		
			Histamine	12	3	1,900	340
			Methylhistamine	7·1	3·3	800	420
			Methylimidazoleacetic acid	24	18	1,100	1,900
			Total imidazoleacetic acid	14	14	0	1,000
49			Free	13	Lost	—	(1,000)
55							

and methylimidazoleacetic acid. In order to gain more information about this we have measured the amounts of [^{14}C]histamine, methylhistamine and methylimidazoleacetic acid in arterial blood, in renal venous blood and in urine during a steady intravenous infusion of [^{14}C]histamine. At the same time assays were also made for free imidazoleacetic acid and imidazoleacetic acid riboside, both products of the action of histaminase on [^{14}C]histamine (Tabor and Hayaishi, 1955; Karjala, Turnquest, and Schayer, 1956). Such an experiment which also included estimation of renal blood flow is illustrated in Table IV.

The concentration of PAH in the two samples of arterial blood taken with an interval of 7 min. was 1.75 and 1.80 mg./100 ml. The corresponding samples of renal venous blood contained 0.55 and 0.60 mg./ml. Thus the arterio-venous difference was 1.20 mg./100 ml. and, since the amount of PAH excreted/min. was 2.33 mg., the blood flow through the kidney calculated according to the Fick principle was 194 ml./min. The concentrations of [^{14}C]histamine and its metabolites in arterial and in renal venous blood are given in Table IV, which also shows the amounts of [^{14}C]histamine and its metabolites removed from the blood by the kidney as calculated from the arterio-venous differences and the figure for renal blood flow obtained from the PAH studies. For comparison the amounts of [^{14}C]histamine and metabolites appearing in the urine have also been included in the Table. The sum of the amounts of [^{14}C]histamine and its metabolites removed from the blood/min. equalled the sum of the amounts excreted in the same time. However, only 18% of the [^{14}C]histamine removed from the blood was found in the urine as histamine. Less methylhistamine was found in the urine than was removed from the blood, whereas the opposite was the case with the methylimidazoleacetic acid. It thus seems probable that some methylhistamine was oxidized to methylimidazoleacetic acid in the kidney. The sum of methylated [^{14}C]histamine derivatives appearing in the urine/min. was greater than the sum of the same derivatives removed from the blood in the same time. There is thus some evidence that methylation took place in the kidney. Little imidazoleacetic acid was removed from the blood and thus the greater part of the imidazoleacetic acid in the urine was probably formed in the kidney. The figures for free imidazoleacetic acid in arterial blood and in urine very nearly equalled those for total imidazoleacetic acid, indicating that the dog did

TABLE V

Male dog, 9 kg. Urine and blood samples collected as described in Table I. Weight of right kidney 22 g. The blood pressure was 100 to 130 mm. Hg.

Time in Min.	Intravenous Infusion of [^{14}C]Histamine 0·5 μg./kg./min.	Collection of Urine	Sampling of Blood	Conc. in Whole Blood of [^{14}C]Histamine and its Metabolites (c./min./ml.)		Arterio-venous Difference in the Concentration of [^{14}C]Histamine and its Metabolites (c./min./ml.)	Extraction Ratio	Amount of [^{14}C]Histamine or Metabolite Excreted by the Kidney/min. (c./min.)
				Arterial	Renal Venous			
0	┌							
20	|	┌						
32	|	|	Histamine	9·8	1·8	8·0	0·8	150
	|	|	Methylhistamine	2·2	1·2	1·0	0·5	150
	|	|	Methylimidazoleacetic acid	17	13	4	0·2	870
44	|	|	Free imidazoleacetic acid	3·5	3·1	0·4	0·1	260
56	┘	┘						

not form imidazoleacetic acid riboside. This is in agreement with the results obtained by Schayer (1956).

The sum of the arterio-venous differences for [^{14}C]histamine and its metabolites in Table IV is about 19 c./min./ml. blood and the sum of the same compounds appearing in the urine/min. is about 3,700 c./min. If these values are used in an estimation of the renal blood flow according to the Fick principle the value obtained is 195 ml./min. The blood flow as calculated from the PAH values was as mentioned above 194 ml./min.

Another experiment comprising of estimations of [^{14}C]histamine and its metabolites in arterial blood, in renal venous blood and in urine during a steady infusion of [^{14}C]histamine is summarized in Table V. As PAH was not given to this dog, the blood flow through the kidney was not calculated. The extraction ratio for [^{14}C]histamine was 0.8. From the estimates for the arterio-venous differences in the concentration of [^{14}C]histamine and its metabolites it may be seen that the methylated [^{14}C]histamine derivatives comprise 40% of the [^{14}C]-labelled compounds removed from the blood, whereas in the urine the methylhistamine and the methylimidazoleacetic acid comprised 70% of the [^{14}C]-labelled substances. This may be taken to indicate that methylation had taken place in the kidney. As judged from the values for imidazoleacetic acid, assayed in the free form only, the greater part of this acid appearing in the urine was formed in the kidney.

DISCUSSION

In the present study the renal extraction and excretion of [^{14}C]histamine during a steady intravenous infusion of [^{14}C]histamine were measured in five dogs. At a concentration of 5 to 15 c./min./ml. arterial blood of [^{14}C]histamine in these experiments, the extraction ratio was 0.7 to 0.8 in dogs which had not been given a histaminase inhibitor. This high extraction ratio could be explained on the assumption that histamine is secreted by the tubular cells in the kidney. Lindahl and Sperber (1956) showed that histamine is excreted by the tubular cells in the kidney of the hen, and a mechanism for the tubular secretion of organic bases has been demonstrated in the dog kidney too (Beyer, Russo, Gass, Wilhoyte, and Pitt, 1950). The relatively small amounts of [^{14}C]histamine appearing in the urine do not, however, lend support to the view that the histamine was secreted to any greater extent by the tubular cells of the kidney. In the two experiments

presented in Tables III and IV, only 14 to 18% of the [^{14}C]histamine removed from the blood by the kidney was excreted in the urine. The clearance values for [^{14}C]histamine calculated in the conventional way are 0.5 to 0.7 ml. whole blood/min./g. of kidney in dogs which had not been given aminoguanidine. The results of the present study indicate that the histamine was taken up from the blood by the tubular cells of the kidney but was not, or only to a small extent, secreted by these cells so that it appeared as histamine in the urine. Emmelin (1951) showed that in cats large quantities of histamine could be taken up from the blood by the kidney and kept there for some time. In our experiments with dogs, where comparatively small doses of histamine were used, there was no evidence for the storage in the kidney of any greater part of the [^{14}C]histamine that had been removed from the blood by the kidney. Evidence was, however, obtained that the histamine was metabolized in the kidney. The canine kidney is richly supplied with histaminase and it seems likely that this enzyme is responsible for the metabolism of some of the histamine in the kidney. This view is supported by the observation here and in previous studies that histaminase inhibitors decrease the capacity of the kidney to remove injected histamine from the blood. Additional evidence for the metabolism of histamine by the renal histaminase was obtained from the experiments presented in Tables IV and V, which indicated that the greater part of the imidazoleacetic acid appearing in the urine during an intravenous infusion of [^{14}C]histamine was formed in the kidney. In this as well as in previous investigations (Lindell and Westling, 1956; Lindell, 1957a and b), it was found that the kidney retained a considerable capacity to remove injected histamine from the blood even after the administration of histaminase inhibitors. The effects of the dose of aminoguanidine used here (12 mg./kg.) on the renal removal of injected histamine were not materially increased by the administration of other histaminase inhibitors (Lindell, 1957a). The methylation of histamine (Schayer and Karjala, 1956) is not inhibited by aminoguanidine and it seems possible that some of the renal removal of injected histamine from the blood is connected with the action of the methylating enzyme. The studies of the amounts of methylhistamine and methylimidazoleacetic acid in arterial blood, in renal venous blood and in urine during an intravenous infusion of [^{14}C]histamine indicated that methylation may take place in the canine kidney and also that methylhistamine may be oxidized in the kidney to methylimidazoleacetic acid.

It should perhaps be pointed out that with a substance such as histamine, which seems to be rapidly metabolized in the kidney, the term clearance may be misleading. The experiment presented in Table IV may serve to illustrate this. The clearance value for [^{14}C]histamine in this experiment calculated in the conventional way would be about 25 ml. whole blood/min. The amount of [^{14}C]histamine removed from the blood by the kidney/min. was 1,900 c./min. as calculated from the mean of the arterio-venous differences in the concentration of [^{14}C]histamine (9.8 c./min.) and the value for renal blood flow, based on the PAH studies (194 ml. blood/min.). Since the mean concentration of [^{14}C]histamine in arterial blood was 14 c./min./ml., it might also be said that 140 ml. blood had been cleared of [^{14}C]histamine by the kidney in one minute.

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METABOLISM OF INJECTED [^{14}C]HISTAMINE IN THE KIDNEY OF THE DOG

BY

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In experiments on anaesthetized dogs, [^{14}C]histamine was given in a steady infusion into one of the renal arteries, while urine was collected from both kidneys separately. The kidney which received the intra-arterial injection of [^{14}C]histamine excreted several times more [^{14}C]-labelled methylhistamine, methylimidazoleacetic acid and imidazoleacetic acid than did the other kidney, indicating that histamine may be inactivated in the canine kidney not only by histaminase but also by the histamine methylating enzyme.

In a previous investigation (Lindell and Schayer, 1958), evidence was obtained that [^{14}C]histamine, given by vein, was taken up by the canine kidney and metabolized there not only by histaminase but also by the enzymes which lead to the formation of methylhistamine {4-(2-aminoethyl)-l-methylimidazole} and methylimidazoleacetic acid (l-methylimidazol-4-ylacetic acid (Schayer and Karjala, 1956). The work reported here is concerned with the fate of histamine injected into the renal artery of the dog.

METHODS

The dogs were anaesthetized with pentobarbitone sodium (30 mg./kg.) given intravenously. Under fluoroscopy, a catheter was guided into one of the renal arteries from a femoral artery (Lindell and Olin, 1957). [^{14}C]Histamine was dissolved in 0.9% saline containing 1 mg. glucose/ml. This solution was injected with a motor-driven syringe through the catheter in the renal artery, at a rate of approximately 1 ml./min. The concentration of [^{14}C]histamine in the solution was such that in the first experiment about 4 μg. histamine base was given/min. In the second experiment the amount was about 1 μg./min. These quantities had no observable effects on the blood pressure measured with a mercury manometer connected to a carotid artery. During the infusion of [^{14}C]histamine urine was collected from polythene tubes inserted into the ureters.

Assay of [^{14}C]Histamine and its Metabolites in Urine.—The [^{14}C]histamine, methylhistamine, methylimidazoleacetic acid and free imidazoleacetic acid were determined with the isotope dilution technique as described by Lindell and Schayer, 1957. The amounts are given in counts/min. above background (c./min.). The c./min. refer to the [^{14}C]-labelled compounds in the form of picrates; 1 μg. of [^{14}C]histamine in the form of the picrate gave 2,040 c./min. in the flow counter (background 19 to 22 c./min.), when non-isotopic histamine carrier corresponding to 40 mg. of histamine base had been used.

RESULTS

The results of two experiments have been summarized in Table I. In the first experiment [^{14}C]histamine was injected into the right renal artery, at a rate of 4 μg./min. The urine collected from the ureters during the infusion was analysed for [^{14}C]histamine and its metabolites and the amounts of these compounds excreted/min. calculated. It may be seen in Table I that the right kidney which received the intra-arterial infusion of [^{14}C]histamine excreted 12 times more methylhistamine than did the left kidney, while the amount of methylimidazoleacetic acid appearing in the urine from the right kidney was nearly three times greater than that in the urine from the left kidney. The amount of free imidazoleacetic acid excreted/min. in the urine from the injected kidney was about 13 times greater than that in the urine from the other kidney. The sum of the differences between the kidneys in the amounts of [^{14}C]histamine and its metabolites excreted/min. was about 5,500 c./min., of which about 1,400 or 25% were in the form of histamine.

In the second experiment [^{14}C]histamine was injected into the right renal artery at a rate of 1 μg./min. As may be seen from the values of the renal excretion of [^{14}C]histamine and its metabolites, about 17 times more methylhistamine was excreted by the injected kidney than by the other kidney. For methylimidazoleacetic acid, the rela-

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TABLE I

EXPERIMENTS ON ANAESTHETIZED DOGS

Urine was collected from each ureter, separately, while [^{14}C]histamine was infused into the right renal artery. In expt. 1, 4 μg./min. was given for 25 min. In expt. 2, 1 μg./min. was given for 20 min.

Expt. No.	Amounts of [^{14}C]Histamine or its Metabolites Excreted/min. During the Infusion of [^{14}C]Histamine (c./min.)				Wt. of Kidney (g.)	Volume of Urine (ml.)
	Histamine	Methylhistamine	Methylimidazole-acetic Acid	Imidazoleacetic Acid		
1. Male dog (8 kg.)						
Injected kidney	1,400	580	1,200	3,100	17	3
Control ,, ...	36	48	420	240	16	4·8
2. Male dog (26 kg.)						
Injected kidney	230	120	140	530	44	14
Control ,, ...	9	7	22	64	44	11

tion between the excretion on the injected side and on the other side was approximately as six is to one. The amount of free imidazoleacetic acid appearing/min. in the urine from the injected side was about eight times greater than that in the urine from the other side. The sum of the differences between the two kidneys in the amounts of [^{14}C]histamine and its metabolites excreted/min. was about 900 c./min., of which 220 or about 25% were in the form of histamine.

DISCUSSION

These results show that, as might be expected, much more [^{14}C]histamine was excreted in the urine from the kidney which received the intra-arterial injection of [^{14}C]histamine. However, metabolites of [^{14}C]histamine were also excreted in much greater amounts on the injected side. The excess of metabolites of [^{14}C]histamine on the injected side was so large that it must be assumed that a considerable amount of the metabolites had been formed in the kidney, which received the intra-arterial infusion. The volumes of the urines collected from each side were approximately equal. It should be mentioned that the catheter used for the intra-arterial injection did not influence the clearance of phenol red either on the catheterized side or on the opposite side (Lindell and Olin, 1957). Also it did not change the renal extraction and excretion of *p*-aminohippuric acid (Lindell, unpublished observation). The possibility that the [^{14}C]histamine was metabolized by the blood in the kidney on the catheterized side seems too unlikely to deserve serious consideration.

That the canine kidney can inactivate histamine is well known since the pioneer investigation of Best and McHenry (1930). Their results have been confirmed by, among others, Steggerda, Essex, and Mann (1935) and Valette, Huidobro, and Cohen (1955). The results in the present study indicate that of the [^{14}C]histamine removed from the blood by the kidney, part (25%) is excreted in the urine as histamine, part is oxidized to imidazoleacetic acid and part is methylated on the ring nitrogen atom remote from the side chain to form a methylhistamine, which seems to have very little biological activity (Lee and Jones, 1949). The excess of methylimidazoleacetic acid excreted on the injected side was probably due to oxidation in the kidney of some of the methylhistamine formed there. The results of the present investigation thus confirm those obtained in the earlier study on the renal removal of injected [^{14}C]histamine in dogs (Lindell and Schayer, 1958).

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CONTINUOUS RECORDING OF ACID GASTRIC SECRETION IN THE RAT

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A method is described for the continuous recording of acid gastric secretion in the rat. The stomach of the rat anaesthetized with urethane is perfused with a dilute sodium hydroxide solution by way of the oesophagus and the *p*H of the fluid emerging from a cannula in the pylorus is registered graphically. In passing through the stomach, the perfusate collects sufficient buffer to act as an approximately linear buffer system over the relevant range, so that the change in *p*H becomes a measure of acid secretion. The preparation is suitable for the bioassay of secretory stimulants. Ten or more drug doses can be administered in succession in one preparation, so that each animal serves as a self-contained assay unit. Intravenous doses of histamine, methacholine, carbachol and acetylcholine produce a graded and reversible stimulation of acid secretion. The secretory effect of histamine is markedly and specifically potentiated by antihistaminases.

The effect of drugs on gastric secretion in the rat has usually been tested in the pylorus-ligated preparation (Roe and Dyer, 1939; Komarov, Shay, Rayport, and Fels, 1944; Shay, Komarov, Fels, Meranze, Gruenstein and Siplet, 1945). In this method only one dose of drug is administered to each animal so that large numbers of rats are required for a quantitative assay. In the present paper a continuous recording method is described which is sufficiently sensitive to allow several doses to be administered in succession to the same preparation so that each animal serves as a self-contained assay unit or sub-unit.

Methods for the continuous recording of the *p*H of the gastric contents have previously been described (Flexner and Kniazuk, 1940; Rovelstad, Owen, and Magath, 1952), but they provided no information on the amount of acid secreted. In order to obtain this information it is necessary to use a perfusate which changes its *p*H when acid is secreted. In the present work we used a solution of dilute sodium hydroxide, the *p*H of which changes, when perfused through the stomach of the rat, by up to three units, according to the amount of acid secreted.

As single intravenous injections of drugs produce a measurable secretion of acid in this preparation, it can be used for the bioassay of secretory stimulants. The stimulant effects of histamine and choline esters, and the potentiating effects of antihistaminases, will be described.

A brief description of the continuous recording method has already been published (Ghosh and Schild, 1955).

METHODS

Two uniform breeds of rats were used, male and female albino rats and hooded rats. Their weights ranged from 150 to 400 g., with an average of 190 g. The animals were not starved, and were anaesthetized by a single intramuscular injection of urethane. The dose of urethane in terms of body weight required for different rats varied slightly; the sensitivity to urethane appeared to depend on seasonal influences. We generally used doses of 0.5 to 0.7 ml./100 g. of 25% solution of urethane; when the first dose failed to anaesthetize in 30 to 40 min., an additional intramuscular dose was given. These doses caused no interference with respiration. After the administration of the anaesthetic, the body temperature fell. In most experiments it was artificially stabilized at 30° by means of a rectal contact thermometer which controlled the heating of the operating table through an electronic relay. The operating table was tilted and was heated from below by a 25 watt electric lamp, and from above by two 40 watt strip lights (tube lamps) fitted with reflectors, one on each side of the table. When the body temperature was checked by a thermometer introduced into the vagina it varied by less than ±0.25°.

Operative Technique.—The trachea was exposed and cannulated. A polythene tube of 11 cm. length and 2 mm. external diameter was passed into the lower

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oesophagus and tied into the oesophagus at the neck excluding the vagus. The jugular veins were then exposed and cannulated with polythene tubes of 1 mm. diameter bevelled at the tip. The abdomen was opened through a midline incision, the pyloro-duodenal junction exposed and a small glass cannula introduced through a cut in the duodenum into the stomach and secured firmly by tying a ligature round the pylorus, care being taken not to include blood vessels within the ligature. The whole stomach was then brought forward and a longitudinal incision about 1¼ in. long was made with an electro-cautery knife along the middle of the anterior surface beginning as near the fundus as possible. Food particles were scooped out carefully with moist cotton-wool, special attention being paid to the fundal region and crevices between the mucous folds. The whole interior of the stomach was washed with cotton-wool soaked in warm saline and the mouth of the cannula was freed of food debris before closing up. The cut edges were then united and secured firmly by means of a continuous suture through the whole thickness of the wall so that no leakage of fluid occurred. Finally, the structures were returned to their proper places and the abdominal wound closed by two or three interrupted sutures with the free end of the cannula projecting. The whole operation lasted about 30 min.

Continuous Recording of Acid Secretion.—The stomach was perfused continuously with a dilute solution of NaOH, the fluid emerging from the pylorus passing over a glass electrode which recorded *p*H continuously. By the time the fluid had traversed the stomach it had collected sufficient buffer to act as an approximately linear buffer system over the *p*H range 6.5 to 4.5 when titrated with 0.1 N-HCl. There was thus an approximately linear relation between *p*H and acidity. The shape of the titration curve is presumably due to buffers of various kinds secreted by or diffusing from the mucosa of the stomach.

In most experiments N/4000-NaOH was perfused through the stomach at a rate of about 1 ml./min. When this solution was collected after having passed through the unstimulated stomach it gave an initial *p*H of the order of 6 to 6.5.

Gastric Perfusion System.—This is shown in Fig. 1. The solution of NaOH was kept in a Mariotte stock bottle which was fitted with a soda-lime tower and suspended above the preparation. The perfusion rate was controlled by a length of capillary resistance tubing connected to the stock bottle. This in turn was connected to a small jacketed warming coil (kept at 30° by a circulating pump) which was joined directly to the oesophageal tube. The capillary was chosen to give a flow of 1 ml./min. with a pressure of 200 cm., and the flow was maintained constant within 0.1 ml./min. The pyloric cannula was connected to a recording glass electrode through a short polythene tube. The glass electrode was placed 15 cm. below the level of the animal; a slight negative pressure was thus exerted, and prevented distension of the stomach.

Optimal conditions for an assay obtained when the perfusate emerging from the pylorus had an initial *p*H of about 6.5 which would decrease to about *p*H 4.0 by the action of stimulant drugs. An initial *p*H of 6.5 could generally be achieved by adjusting the perfusion rate within the limits of 1 to 2 ml./min. through variation in perfusion pressure, without changing the concentration of NaOH from the standard molarity of N/4000. In some experiments N/2000 or N/8000-NaOH was used.

The recording glass electrode was made of a lithium glass membrane; it had a diameter of 5 mm. and was fitted into a U-shaped glass container with a capillary lumen. One limb of the U-tube was slightly dilated to hold the glass electrode, and at this point the porous plug of the reference electrode was attached. The dead space within the glass container was 0.2 ml., and the total dead space from the gastric cannula to the bulb 1.3 ml. The whole assembly was held by clamps and a stand in such a way that the reference electrode was at a level 12 in. higher than the glass electrode. As a result of this pressure gradient, the KCl solution diffused slowly and steadily

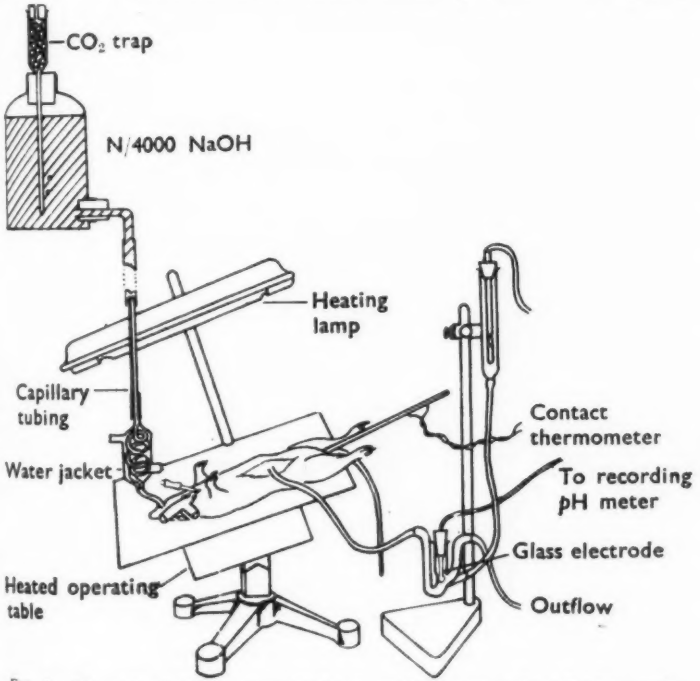

FIG. 1.—Stomach perfusion assembly for a continuous recording of acid secretion in the rat.

through the porous plug serving as an efficient salt-bridge connexion.

The electrodes were connected to a direct reading *p*H meter (Electronic Instruments Ltd., Model 23A) and thence to a circular ink recorder (Fielden's servograph recorder). There was no appreciable drift or lag in the apparatus and the stability of the recorder was within 0.1 *p*H unit. In the illustrations the record has been transformed to rectilinear co-ordinates. Drug responses have been expressed in terms of the maximum deflexion of the *p*H record from the base line.

All drugs were administered intravenously. The first injection was usually made 10 min. after completing the operation. Drugs were injected in a volume of 0.1 to 0.4 ml. followed by a washing injection of 0.1 ml. saline. The concentration of histamine acid phosphate is expressed in terms of the base, and that of the choline esters in terms of the hydrochlorides.

Results

The Effect of Histamine

A typical effect of histamine on acid secretion is shown in Fig. 2. Although the drug was administered by a rapid intravenous injection, it produced a delayed and gradually increasing effect. The *p*H began to fall 4 min. after the injection and reached the lowest point 13 min. later. Only a small fraction of this delay was attributable to mechanical lag in the perfusion system. A rough estimate of this lag could be obtained by injecting acid into the oesophageal tube ; when a dose of

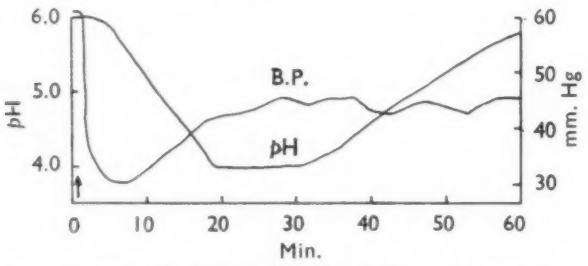

FIG. 2.—Effect of intravenous injection of 100 μg. histamine (at arrow) on blood pressure and *p*H of stomach perfusate. In this and the following illustrations rats anaesthetized with urethane and kept at 30° were used. Ordinates: *p*H of stomach perfusate on the left and blood pressure on the right.

0.1 ml. 0.1 N-HCl was so given, it produced a sharp fall of *p*H at the recording electrode within 40 sec., followed by 90% recovery in 2 min. A record of blood pressure taken simultaneously with the *p*H record in Fig. 2 shows that the two responses have a different time course. The blood pressure fell almost immediately after the intravenous injection and started recovering long before the secretory effect had reached its maximum.

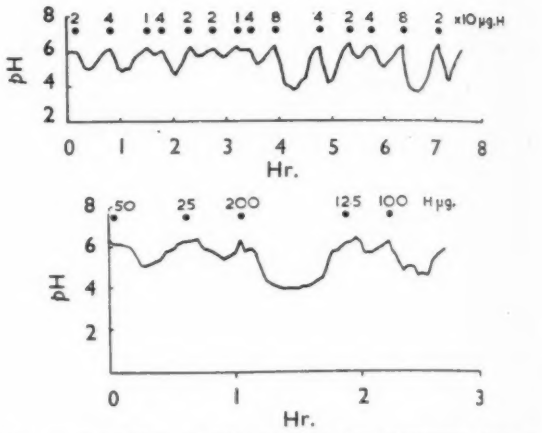

FIG. 3.—Stimulation of acid secretion by intravenous doses of histamine (H). Graded responses were obtained in one preparation with doses of 10 to 80 μg. and in the other with 12·5 to 200 μg. histamine.

Since the effect of an intravenous dose of histamine lasted only 40 to 60 min. several successive doses could be given in the course of an experiment lasting several hours. In the experiments illustrated in Fig. 3, doses over an 8-fold and 16-fold range given in random succession produced graded effects. Repeated administrations of the same dose usually produced constant effects as shown in Fig. 4.

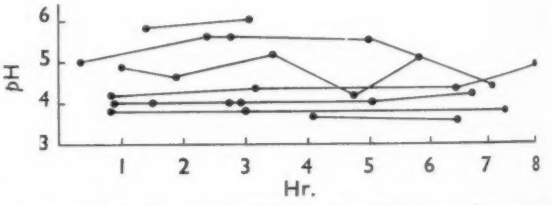

FIG. 4.—Effect of repeated administration of the same intravenous dose of histamine on acid secretion. Responses obtained in the same preparation are joined. The response to the first dose was omitted in each case.

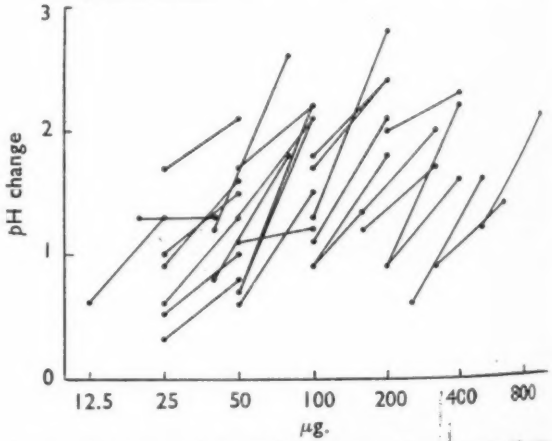

FIG. 5.—Effect of two doses of histamine on acid secretion in 30 rats.

There was a great deal of variation in the sensitivity of the rats. In some, 25 μg. histamine produced an average secretory response ; in others as much as 500 μg. was required, and about 10% failed to respond to doses of 1 mg. Fig. 5 shows the responses obtained with two graded doses of histamine in 30 rats. The average sensitivity of the preparations varied about 20-fold, but in each (except one) the effect increased with dose.

Effect of Body Weight.—Fig. 6 shows the histamine dose/100 g. of body weight required for a standard secretory effect when plotted against body weight. There is some evidence of a possible correlation between the two variables, suggesting that small rats are rather more sensitive to the secretory stimulus of histamine than large rats.

Effect of Body Temperature.—When the body temperature was changed by altering the external heat supply, the histamine response was consistently decreased by warming and increased by cooling. In one experiment, histamine produced a large effect at 30°, progressively smaller effects at 36°, and another large effect at 30°. In another experiment two large responses occurred at 30°, followed by a small response at 34°. Three further experiments are summarized in Fig. 7. In each, the magnitude of secretory response changed in a direction opposite to the body temperature, and this effect was reversible.

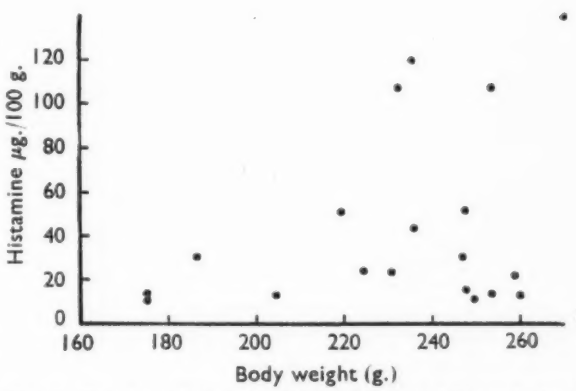

FIG. 6.—The dose of histamine/100 g. of body weight required to produce a standard secretory effect plotted against the body weight.

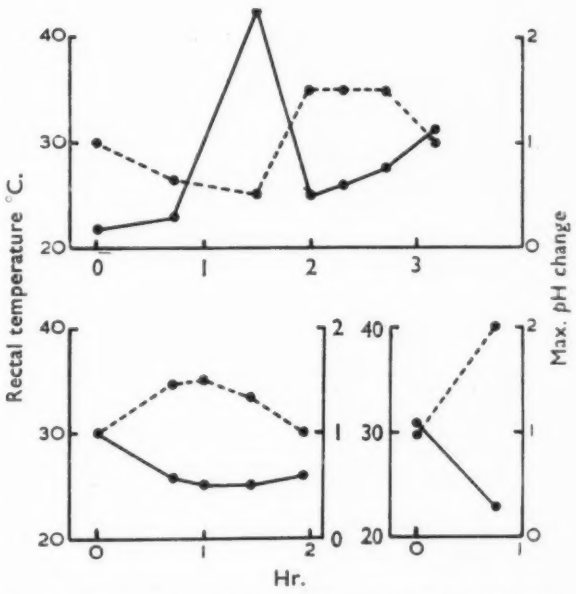

FIG. 7.—Effect of temperature on acid secretion induced by intravenous injections of a constant dose of histamine. Summary of three experiments. Temperature, broken line; *p*H, solid line.

Choline Esters

Acid secretion could be induced by intravenous injection of carbachol, methacholine, and acetylcholine. Acetylcholine was relatively inactive and tended to produce toxic side reactions culminating in respiratory arrest. It was nevertheless possible to record stimulation of secretion by acetylcholine several times. The effect of acetylcholine was potentiated by neostigmine. The degree of potentiation was related to the dose of neostigmine and the potentiation persisted for some time.

Methacholine was more active than acetylcholine. Graded responses were obtained within the range of 0.1 to 20 μg. The sensitivity to methacholine often increased in the course of an experiment. In one experiment the sensitivity increased nearly 100-fold in the course of 4 hours.

Carbachol proved a very powerful stimulant of secretion. In doses of 0.02 to 2 μg., it produced graded effects, the intensity and duration of which

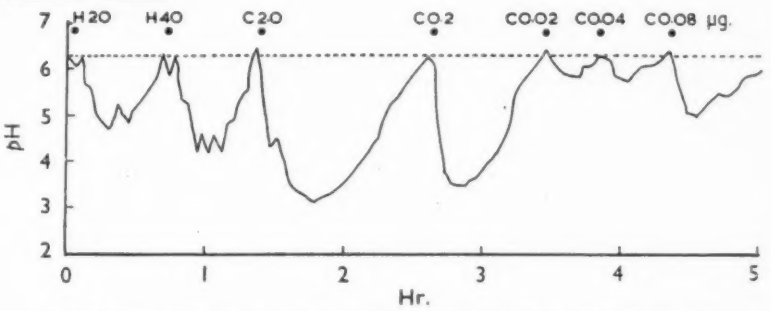

FIG. 8.—Secretory effects of graded doses of histamine (H) and carbachol (C).

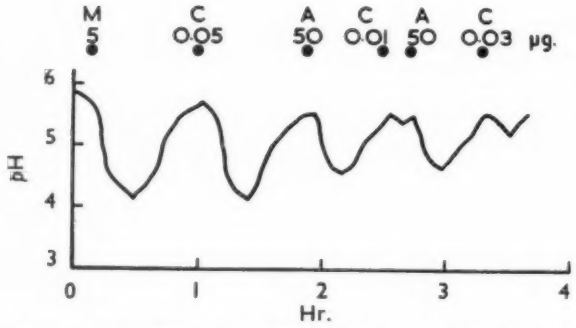

FIG. 9.—Comparison of secretory effects of methacholine (M), carbachol (C), and acetylcholine (A).

increased with the dose as shown in Fig. 8. Weight for weight, carbachol was considerably more active than histamine.

A direct comparison of the secretory effects of these three choline esters is shown in Fig. 9. In this experiment carbachol was 100 times as active as methacholine and about 1,000 times as active as acetylcholine. Table I summarizes the activity ratios obtained in six assays. Although the activity ratios varied in different preparations carbachol was obviously the most active, and acetylcholine the least active of the three compounds. The activity ratios for acetylcholine hold only for small

TABLE I

ACTIVITY RATIOS OF ACETYLCHOLINE, METHACHOLINE AND CARBACHOL

Drug Ratios	Exp. No.	Estimated Activity Ratios	
Methacholine / Acetylcholine	168	>10	<20
		>10	<40
	179	=200	>50
	183		>10
	191		>25
Carbachol / Acetylcholine	139		>1,000
	168	>10	<100
	179		>500
	183	>1,000	<1,600
	184	>250	=500

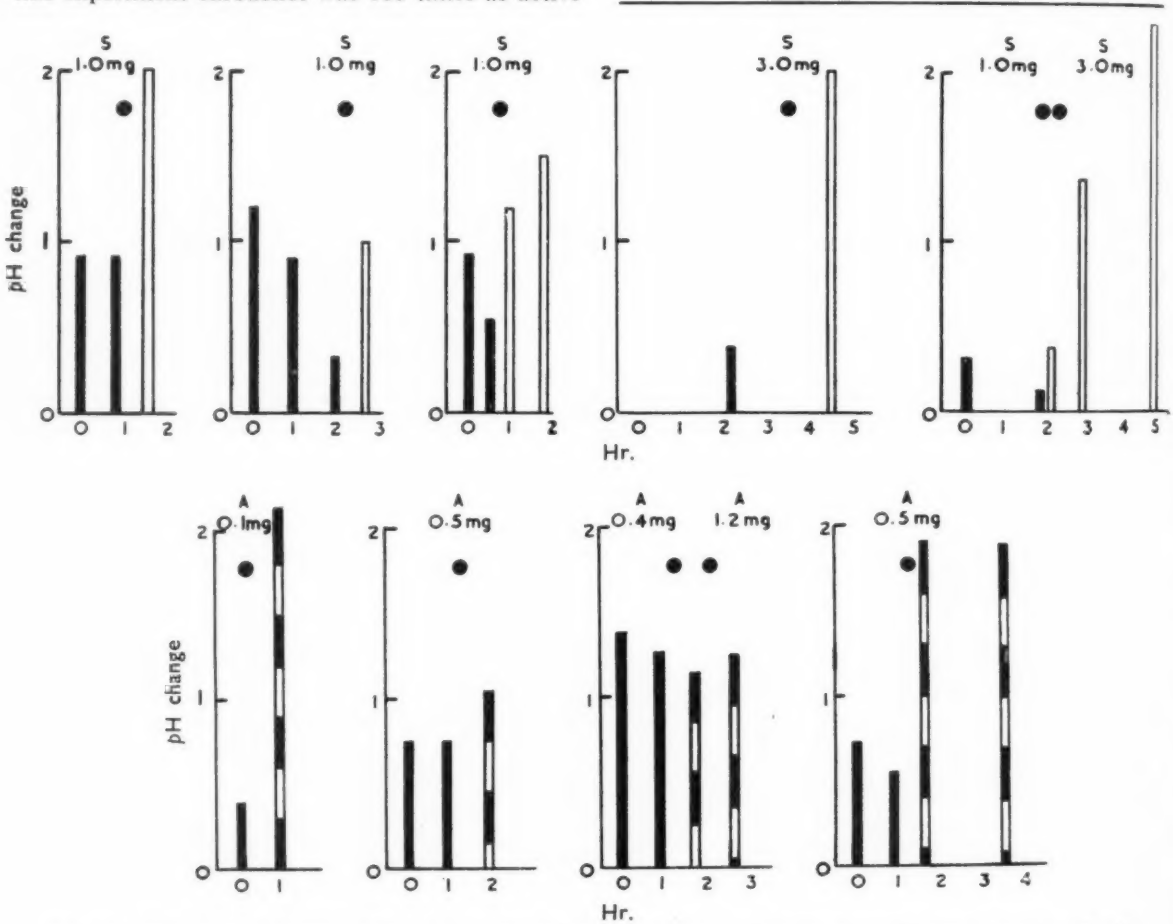

FIG. 10.—Potentiation of histamine secretion by semicarbazide (S) and aminoguanidine (A) in two different experiments. The dose of histamine in each experiment was kept constant.

or medium secretory effects, since doses of acetylcholine producing large effects were not administered owing to their toxicity.

The Effect of Antihistaminases

Two inhibitors of histaminase, aminoguanidine (Schuler, 1952) and semicarbazide (Zeller, 1942), were used. The antihistaminases were administered intravenously shortly before or together with the test drug, in doses in which they produced no secretion on their own.

Both aminoguanidine and semicarbazide produced a strong potentiation of histamine secretion. Semicarbazide was administered in five experiments in doses of 1 and 3 mg., some experiments involving several injections. The results are summarized diagrammatically in Fig. 10*a*, which shows that each time the effect of histamine was potentiated. Aminoguanidine was administered in five experiments in doses ranging from 0.1 to 1.2 mg. The results with aminoguanidine are summarized in Fig. 10*b*, which shows potentiation of histamine in all experiments except one.

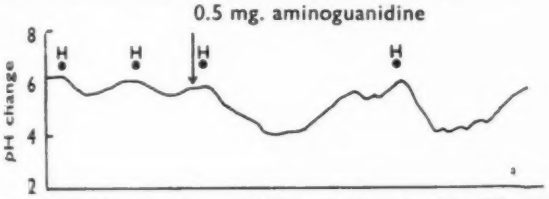

FIG. 11.—Persistence of potentiation of histamine (H = 400 μg.) secretion by aminoguanidine.

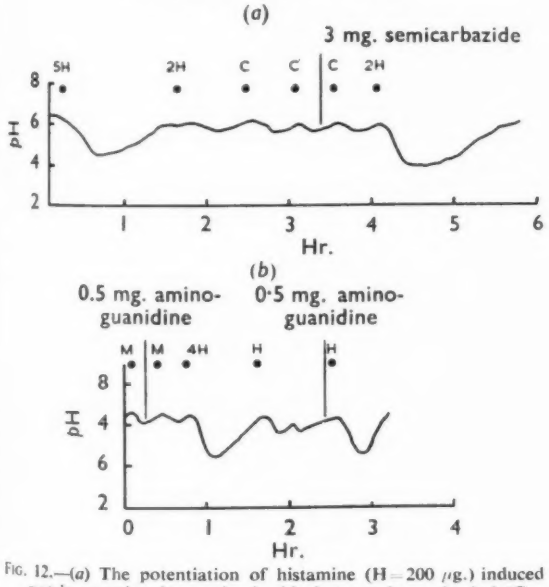

FIG. 12.—(*a*) The potentiation of histamine (H = 200 μg.) induced gastric secretion by semicarbazide but not by carbachol (C = 0·2 μg.) secretion. (*b*) Aminoguanidine potentiation of histamine secretion (H = 12·5 μg.) but not of methacholine (M = 50 μg.) secretion.

Potentiation by a single dose of antihistaminase sometimes persisted during subsequent injections of histamine; in one experiment a second dose of histamine given 1 hr. after the administration of aminoguanidine produced a greater effect than the first dose given with the aminoguanidine. An example of persistent potentiation is shown in Fig. 11. Two consecutive doses of 400 μg. histamine produced only small effects, but the same dose of histamine was still potentiated when given 2 hr. after 500 μg. of aminoguanidine. Neither methacholine- nor carbachol-induced secretion was appreciably increased by antihistaminases. Fig. 12 shows experiments in which antihistaminases were tested with histamine and with choline esters in the same preparation. Fig. 12*a* shows no potentiation of carbachol by semicarbazide, but a strong potentiation of histamine at a later stage. Fig. 12*b* shows no potentiation of methacholine by aminoguanidine, but potentiation of histamine. In two other experiments, antihistaminases produced a slight potentiation of the effects of choline esters; but this was variable and not comparable in intensity with the potentiation of histamine.

Discussion

The method of continuous recording of *p*H has obvious limitations. It measures total acid secretion, but gives no indication of the volume and acidity of the gastric juice, and the artificial conditions under which the animal is maintained, namely the low body temperature, anaesthesia and alkalinity of the stomach perfusate, may affect both acid and mucus secretion. On the other hand, the method has certain advantages for bioassay work: it is sensitive and follows the time course of secretion closely, so that successive doses of drugs can be administered as soon as the effect of a previous dose subsides.

In the early stages of this work distilled water or saline was used to perfuse the stomach; but it soon became apparent that these fluids were unsuitable since they gave initial *p*H values of 4 to 5 which were little affected by injections of histamine. Perfusion of the stomach with a linear buffer also proved unsuitable. A linear buffer has the theoretical advantage of a more truly linear relationship between acidity and *p*H, but the concentration-action curve becomes flat.

The procedure for recording *p*H continuously, which may be applicable to other species, seemed to depend for its success on the following points: (1) Low body temperature. Anaesthetized rats survived better at 30° than at 37° or even at

34°, and both the circulation and respiration were less liable to fail. (2) Cleaning of stomach. The difficulties of clearing the rat stomach of food residues by fasting are well known. We found that after 24 hr. fasting only about 80 to 90% of animals were free of food residue and that even longer fasting periods were frequently unsuccessful. When the unopened stomachs of these animals were perfused through the oesophagus it was not possible to clear the fundus completely of food residue and the secretory activity appeared very irregular. Opening of the stomach by a wide incision over the major curvature, and scooping out the contents, obviated these difficulties and produced surprisingly little interference with gastric secretion. There was no leakage from the operation wound, and the animals were not devitalized by fasting. (In some recent experiments the unopened stomach was washed out through a thin catheter introduced by way of the pylorus cannula. Satisfactory recordings were obtained by this method.) (3) Urethane anaesthesia. Since anaesthetics affect gastric secretion (Schachter, 1949), it is essential in any bioassay to keep the depth of anaesthesia constant. This cannot be achieved by anaesthetics such as pentobarbitone, which have a short duration of action and must therefore be administered repeatedly. Urethane when given in a single dose produced an apparently unchanged anaesthesia for at least 8 hr.

The Effect of Histamine

When an intravenous dose of histamine is given to rats the blood-pressure response is immediate, but the secretory response has a characteristic latent period and reaches a maximum only after about 15 min. This delay, which is surprising since the plasma concentration of histamine must be at its peak immediately after the injection, could be explained in two ways. It might be due to the slow rate at which secreted acid reaches the surface of the stomach. A second dose of histamine would then be expected to produce less delay since the necks of the glands would still be filled with secretion, but, in fact, the response to a second dose is equally delayed. Alternatively, the delay may indicate that the oxyntic cell responds only to a protracted stimulus. It is known that gastric secretion cannot readily be induced in the dog by single intravenous injections of histamine (Popielski, 1920; Rothlin and Gundlach, 1921; Ivy and Javois, 1925; but see Schofield, 1957), but only by slow infusions, and it may be that in rats a single intravenous dose of histamine circulates for some time in the blood stream and acts like a slow infusion. Single intravenous doses of histamine may produce a better gastric secretory response in rats simply because of their high tolerance for histamine, so that it is possible to administer large doses which take a long time to disappear from the blood stream.

The Effect of Antihistaminases

Antihistaminases produce a specific potentiation of histamine secretion in the rat. This agrees with their specific potentiating effect in other species and preparations (Arunlakshana, Mongar and Schild, 1954; Westling, 1956) but contrasts with their effect on gastric secretion in dogs. Sircus (1953) and Ivy, Lin, Ivy, and Karvinen (1956) found in dogs that antihistaminases potentiate not only histamine secretion but also secretion induced by carbachol, insulin and feeding. These results were interpreted in terms of a release and subsequent potentiation of histamine from the gastric mucosa by these various stimuli. This would support the theory of histamine as the final common path for gastric secretion (MacIntosh, 1938; Babkin, 1938); but against this interpretation is the fact that the gastric mucosa contains no histaminase to account for the potentiation. In rats, however, the antihistaminases potentiate histamine secretion selectively, and it is thus unnecessary to postulate that they act on the gastric mucosa; they presumably inhibit histaminase elsewhere in the body, for example in the kidney and intestine (Waton, 1956), and, in this way, prolong the circulation time of injected histamine.

Direct Assays

Direct assays of secretory stimulants can be performed in this preparation by injecting the drugs intravenously and matching their effects. Carbachol was shown to be 50 times as active as methacholine and 1,000 times as active as acetylcholine. When the choline esters were injected intravenously they exhibited the same sort of latent period and gradually increasing secretory effects as histamine; it would thus seem that the delay in response observed with histamine is not a characteristic of the drug but of the secretory cell. The high secretory activity of carbachol agrees with results obtained in other species; in the rat it is presumably due, partly at least, to stimulation of ganglia since it was found (unpublished observations) to be reduced by hexamethonium. The low secretory activity of acetylcholine in the rat also agrees with results in other species (Necheles, Mortel, Kosse and Neuwelt.

1938; Uvnas, 1948; Morton and Stavraky, 1949; Pevsner and Grossman, 1955) and can be explained by a combination of two factors, firstly the slowness of the response of the oxyntic cell, and secondly the action of cholinesterase. In the presence of neostigmine the secretory effects of acetylcholine were indeed strongly potentiated.

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THE RELATIONSHIP BETWEEN 5-HYDROXYTRYPTAMINE AND ADENOSINE TRIPHOSPHATE IN BLOOD PLATELETS

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In normal platelets a proportionality was found between the amount of adenosine triphosphate (ATP) and amount of 5-hydroxytryptamine (5-HT) in platelets both before and after incubating them in plasma to which 5-HT had been added. In patients receiving reserpine and in others with myeloid leukaemia, the amount of 5-HT in the platelets and the uptake of 5-HT by them were depressed, while the amount of ATP was normal. The possibility that ATP is involved in the accumulation of 5-HT by platelets is discussed.

Hardisty and Stacey (1957) showed that, in myeloid leukaemia and some other diseases of the blood, the amount of 5-hydroxytryptamine (5-HT) in the platelets is reduced. In some cases this was associated with an impaired capacity of the platelets to take up 5-HT when they were incubated in plasma to which 5-HT had been added. Further, it has been shown (Born, Ingram, and Stacey, 1956; Born and Gillson, 1957) that the amount of 5-HT in platelets is proportional to the amount of adenosine triphosphate (ATP) which they contain. This paper reports observations on the amounts of 5-HT and ATP in platelets obtained from normal people, from patients suffering from myeloid leukaemia and from others treated with reserpine. Some of the experiments have been briefly reported (Born *et al.*, 1956).

METHODS

The methods described by Hardisty and Stacey (1955) were used for the preparation of platelet-rich plasma and for the determination of the 5-HT content of the platelets and of their ability to take up 5-HT added to the plasma. ATP was extracted from platelets with trichloracetic acid and estimated by the method of Strehler and Totter (1954), using firefly luminescence.

RESULTS

Platelets obtained from 15 normal persons were analysed for ATP and 5-HT. The results are recorded in Fig. 1, in which the amounts of these two substances are plotted against one another. Although there is considerable scatter, there is a rough proportionality between the amounts of 5-HT (y) and ATP (x) in the platelets which is expressed by the equation $y=0.01+0.023x$ ($r=0.67$, $P<0.01$).

Fig. 2 shows that after platelets from the same people had been incubated with 5-HT the proportionality became closer. The corresponding equation is $y=0.035+0.094x$ ($r=0.78$, $P<0.001$). The mean ratios of the number of ATP molecules to the number of 5-HT molecules in the platelets before and after incubation with 5-HT are given in Table I, together with the ranges and standard errors.

Ten patients with myeloid leukaemia were investigated. Of these, five had received no treatment

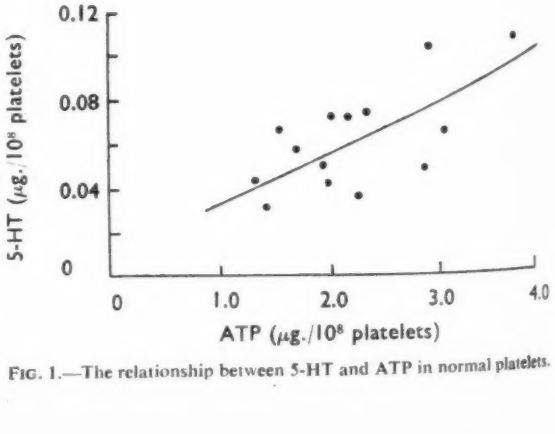

FIG. 1.—The relationship between 5-HT and ATP in normal platelets.

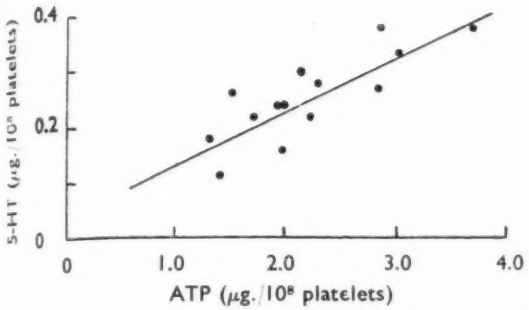

FIG. 2.—The relationship between 5-HT and ATP in normal platelets after incubation at 37° in plasma containing added 5-HT.

and five had started treatment with busulphan. Both groups showed the diminished platelet 5-HT and impaired ability to absorb 5-HT previously described, but the mean concentration of ATP in the platelets of these patients did not differ significantly from the normal, and in 8 of 10 it was within normal range. These changes are reflected in the molecular ratio ATP/5-HT, which is significantly higher than in the normals.

The 5-HT content of platelets and their ability to absorb 5-HT is reduced by the administration of reserpine. This has been shown for human platelets by Haverback, Shore, Tomich, and Brodie (1956) and by Hardisty, Ingram, and Stacey (1956) and is apparent from the results recorded in Table I. One of the subjects of the present investigation was taking 0.25 mg. reserpine by mouth three times a day, and four had received a single dose of 1 mg. intramuscularly 48 hr. before the blood was taken. The mean platelet ATP is not significantly different ($P>0.1$) from the normal, but the ratio ATP/5-HT in the platelets is greatly raised.

DISCUSSION

The results show that, in normal human platelets, the amount of 5-HT is proportional to the amount of ATP, both when the platelets are freshly isolated from the blood and after they have been allowed to take up 5-HT *in vitro*.

Recent experiments have strongly suggested that, in the intracellular granules of the adrenal medulla, the catechol amines are bound to ATP by ionic bonds (Blaschko, Born, D'Iorio and Eade, 1956). It is interesting to consider whether the proportionality between the amounts of 5-HT and ATP in platelets indicates that a similar mechanism brings about the accumulation and binding of 5-HT in the platelets. Some observations support this idea. One is that, when platelets are present in plasma which is clotting, the ATP in the platelets rapidly breaks down and at the same time 5-HT is released from them (Born, 1956). Another is the remarkably close correspondence between the number of molecules of ATP in platelets and the maximum number of molecules of 5-HT which platelets take up under the best experimental conditions (Born and Gillson, 1957)—namely, one to two molecules of 5-HT for each molecule of ATP.

However, it is possible that the variations in the amounts of 5-HT and ATP might both result from changes in a third factor, such as the platelet volume. In the experiments reported here we found that the concentration of ATP in normal platelets varied 2.8-fold, whereas their volume varied only 1.2-fold (Hardisty and Stacey, 1955). This makes it unlikely that platelet volume determines the amount of ATP which the platelets contained.

Our results show that, in patients treated with reserpine and in others suffering from myeloid leukaemia, the amount of 5-HT that the platelets will hold is reduced while the amount of ATP in them remains the same. Two possible explanations of this must be kept in mind. If 5-HT is bound to ATP in platelets this union might be blocked in these people, or alternatively it may be that there is at least one other factor involved in the accumulation of 5-HT by platelets and that it is through this that reserpine acts and which is possibly defective in myeloid leukaemia.

TABLE I

PLATELET 5-HT AND ATP BEFORE AND AFTER INCUBATION OF PLATELETS IN PLASMA CONTAINING ADDED 5-HT

The *numerals* in *italics* are significantly different ($P<0.01$) from the corresponding normal figures. The numerals in Roman type are not significantly different ($P>0.1$) from the corresponding normal.

	No. of Subjects	ng. 5-HT/10⁸ Platelets		μg. ATP/10⁸ Platelets	Molecular Ratio ATP/5-HT	
		Initially	After Incubation with 5-HT		Initially	After Incubation with 5-HT
Normal	15	**60·0±5·1**	**242±21**	**2·2±0·17**	**12·8±0·9**	**3·2±0·24**
Myeloid leukaemia (a) untreated	5	*11·8±3·0*	*95±12*	2·1±0·49	*47·4±9·2*	*8·6±1·7*
" " (b) treated	5	*21·0±5·1*	*110±30*	1·9±0·38		
Reserpine treated	5	*5·4±0·6*	*39±10·5*	2·9±0·35	*186*	*27±5·3*

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INHIBITION BY VARIOUS SUBSTANCES OF OEDEMA FORMATION IN THE HIND-PAW OF THE RAT INDUCED BY 5-HYDROXYTRYPTAMINE, HISTAMINE, DEXTRAN, EGGWHITE AND COMPOUND 48/80

BY

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Of the substances studied for their inhibitory action of the oedema formation in the hind-paw of the rat, the most active was a new derivative of phenothiazine, related to chlorpromazine. It is methotrimeprazine {10-(3-dimethylamino-2-methylpropyl)-2-methoxyphenothiazine ; 7044 RP}. This substance possesses potent antihistamine and anti-5-hydroxytryptamine properties, and doses of 0.25 mg./kg. inhibited the blueing and swelling of the foot after injections of histamine, 5-hydroxytryptamine, eggwhite, dextran or compound 48/80 into the feet of rats with sulphan blue (Evan's blue) dye in their circulation. The results indicate that both histamine and 5-hydroxytryptamine play rôles in increasing capillary permeability in the rat.

It has been shown by Rowley and Benditt (1956) and Parratt and West (1957) that the anaphylactoid reaction produced in rats by injections of eggwhite and dextran is mediated chiefly through the release of 5-hydroxytryptamine. This has been achieved by using drugs which deplete the tissues only of their 5-hydroxytryptamine or block specifically the action of 5-hydroxytryptamine. The purpose of the present experiments is to show that a number of other drugs, which are antagonists of 5-hydroxytryptamine, reduce or prevent the oedema induced by eggwhite and dextran in the rat hind-paw. Antagonism of the oedema induced by compound 48/80, histamine and 5-hydroxytryptamine has also been studied.

METHODS

Groups of 4 to 6 female albino rats (100 to 150 g.) were used in all experiments. Subcutaneous injections of 0.1 ml. of each oedema-producing substance were made into the hind-paws to study the vascular changes after each animal had received an intravenous dose of sulphan blue (Evan's blue) dye (2 mg.) 30 min. previously. Drugs used to inhibit the oedema formation were given intravenously with the dye, except for adrenaline and noradrenaline which were administered subcutaneously at the same time as the intravenous dye. The following solutions of the oedema-producing substances were used: 5-hydroxytryptamine (5 μg./ml.), histamine (1 mg./ml.), dextran (60 μg./ml.), fresh eggwhite (0.5% v/v solution), and compound 48/80 (10 μg./ml.). These concentrations produced nearly maximal oedema of approximately equal severity. Injections of normal saline into some paws served as controls, but the very slight swelling and blueing which were produced in these areas subsided in about 1 hr. when the degree of oedema was assessed. The measurement of the oedema reaction has been described in detail (Parratt and West, 1957). Briefly, we have recorded the degree of swelling (oedema) and of blueing (leakage of plasma proteins into the tissue spaces) on a relative scale from 0 to +++. By allotting marks to each degree (+=2, ++=4, +++=6), the results may be expressed as % of the maximal possible effect (+++) for the dose chosen for each substance. On this system of measurement, a difference of 20% is within the error of the test. The values shown in the Tables are the means of 3 different experiments, separate records of the oedema and blueing being assessed to each rat and the scores then averaged. Usually, oedema and blueing go hand in hand, though this is not always so.

The following drugs have been tested for inhibitory action: 2-bromolysergic acid diethylamide (BOL 148), mepyramine maleate, phenindamine, thenalidine tartrate (1-methyl-4-*N*-then-2′-ylanilinopiperidine tartrate), 5-methoxy-2-methyltryptamine, 1-benzyl-5-methoxy-2-methyltryptamine, adrenaline tartrate and noradrenaline tartrate, and the phenothiazine derivatives—promethazine hydrochloride (3277 RP), chlorpromazine hydrochloride (4560 RP), methylpromazine (4627 RP), 4670 RP {2-chloro-10-(3-pyrrolidin-1′-ylpropyl)phenothiazine}, trimeprazine

(6549 RP) and methotrimeprazine (7044 RP). All the values in the Tables refer to the bases.

Results

BOL 148.—We have previously shown that intravenous doses of 4 mg./kg. of this substance completely prevented oedema formation by 5-hydroxytryptamine, dextran, eggwhite and compound 48/80, leaving that by histamine unchanged. When the dose was reduced to 1 mg./kg., the 5-hydroxytryptamine response was still completely inhibited, but those of the other three substances were only reduced (Table I). This result indicates that BOL 148 is an effective antagonist of exogenous 5-hydroxytryptamine when doses of 1 mg./kg. are used, but larger doses are needed to prevent the action of released 5-hydroxytryptamine.

Mepyramine and Phenindamine.—These two antihistamine drugs were weak in inhibiting the oedema formation by most agents. For example, doses of 10 mg./kg. of mepyramine did not affect the 5-hydroxytryptamine response and only reduced the responses of the other agents (Table I). This reduction, however, probably indicates an inhibitory action on released histamine in the cases of dextran, eggwhite and compound 48/80, since these three substances are capable of releasing both histamine and 5-hydroxytryptamine. Phenindamine was a slightly stronger inhibitor of the oedema response than was mepyramine, yet it is generally considered to be a less potent antihistamine drug. On the other hand, phenindamine is a more potent antagonist of 5-hydroxytryptamine on isolated tissue than is mepyramine, and the combined antagonistic actions of phenindamine probably account for the full inhibition of the dextran and eggwhite responses found at dose levels of 8 mg./kg.

Table I

THE EFFECTS OF INTRAVENOUS DOSES OF ANTAGONISTS ON THE LOCAL OEDEMA REACTION PRODUCED IN RATS BY 5-HYDROXYTRYPTAMINE (5-HT), HISTAMINE, DEXTRAN, EGGWHITE, AND COMPOUND 48/80

Responses measured in terms of the maximal (100%) found in saline-treated rats.

Antagonist		5-HT	Histamine	Dextran	Eggwhite	Compound 48/80
Name	Dose (mg./kg.)					
BOL 148 ..	1	13	100	39	41	42
	2	16	100	17	21	42
	4	10	100	9	20	18
Mepyramine ..	4	100	70	100	100	100
	10	82	42	42	42	42
Phenindamine	4	100	70	75	87	100
	8	70	30	10	20	65
Thenalidine ..	5	100	80	42	100	85
	10	54	52	13	37	85
	15	33	54	4	8	35

Thenalidine.—This substance has been shown by Rothlin and Cerletti (1955) to possess antihistaminic properties as well as marked inhibitory effects on increased capillary permeability shown in various forms of experimental oedema produced by dextran or eggwhite. In the present experiments, it was more effective against dextran and eggwhite than it was against either 5-hydroxytryptamine, histamine or compound 48/80 (Table I). Thenalidine, like phenindamine, possesses some anti-5-hydroxytryptamine properties.

Antimetabolites of 5-Hydroxytryptamine.—The two agents, 5-methoxy-2-methyltryptamine and 1-benzyl-5-methoxy-2-methyltryptamine, used are the most powerful antagonists of 5-hydroxytryptamine known to be effective orally in dogs. Yet, with one exception, maximally tolerated doses (10 mg./kg.) of these substances did not markedly influence the oedema production by either of the agents. The exception was the oedema induced by

Table II

THE EFFECTS OF DOSES OF ANTIMETABOLITES OF 5-HYDROXYTRYPTAMINE (5-HT) AND CERTAIN SYMPATHOMIMETIC AMINES ON THE LOCAL OEDEMA REACTION PRODUCED IN RATS BY 5-HYDROXYTRYPTAMINE, HISTAMINE, DEXTRAN, EGGWHITE AND COMPOUND 48/80

Responses measured in terms of the maximal (100%) found in saline-treated rats.

Antagonist			5-HT	Histamine	Dextran	Eggwhite	Compound 48/80
Name	Route	Dose (mg./kg.)					
5-Methoxy-2-methyltryptamine	Intravenous	4	100	100	100	100	83
	,,	10	70	100	90	76	76
1-Benzyl-5-methoxy-2-methyltryptamine ..	,,	4	88	100	72	84	96
	,,	10	78	84	52	84	84
Adrenaline	Subcutaneous	1	54	84	41	39	65
	,,	2	54	42	18	8	50
	,,	4	20	55	20	8	20
Noradrenaline	,,	10	38	80	38	58	75

dextran which was reduced by the benzyl derivative to about 50% of the maximal response (see Table II).

Sympathomimetic Amines. — Adrenaline was found to be a potent inhibitor of the anaphylactoid reaction of dextran and eggwhite, doses of 2 mg./kg. completely preventing the response. Yet such doses were not sufficient to antagonize completely the effects of 5-hydroxytryptamine, histamine or compound 48/80. If this difference was due to the vasoconstrictor action of adrenaline, then noradrenaline should be more active than, or at least as active as, adrenaline. But the results shown in Table II indicate that noradrenaline is only about 1/10 as active as adrenaline in reducing the oedema production.

Phenothiazine Derivatives. — The chemical structures of the derivatives tested are shown in Fig. 1. Promethazine has been tested previously at a dose of 4 mg./kg. and found to reduce considerably the responses of dextran and histamine.

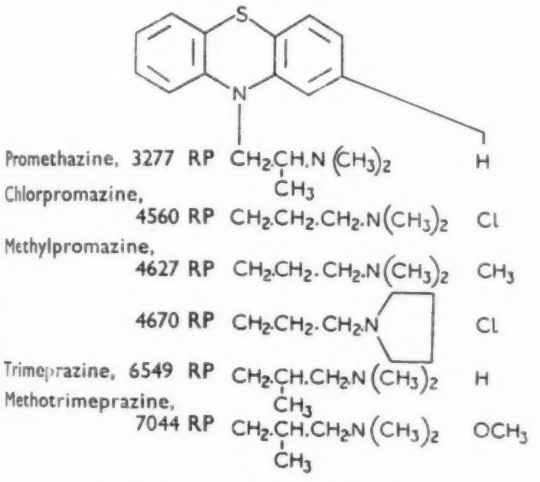

FIG. 1.—Chemical structure of derivatives of phenothiazine.

Chlorpromazine, methylpromazine and 4670 RP were included in this study since Viaud (1954) has reported that these three substances are very effective in protecting rats against dextran-induced oedema. This author linked this protective action of these substances with their antihistamine action. Trimeprazine and methotrimeprazine are newer derivatives which have also been found in screening tests to be very potent in this action.

All six derivatives were tested at various dose levels against oedema induced by the five agents (Table III). Promethazine in a dose of 8 mg./kg. completely prevented all the responses whereas 4 mg./kg. markedly reduced the oedema produced by dextran, eggwhite, compound 48/80 and histamine. Chlorpromazine was effective against dextran and eggwhite at much smaller doses (0.5 mg./kg.), so confirming the results of Benditt and Rowley (1956). Larger doses, however, were required in our experiments to reduce the responses of 5-hydroxytryptamine, histamine and compound 48/80. Methylpromazine was effective at a dose of 1 mg./kg. against histamine, dextran, eggwhite, and compound 48/80, but much less so against 5-hydroxytryptamine. This latter result shows that histamine plays a rôle (even if only a minor one) in the anaphylactoid reaction. A similar set of results was obtained with trimeprazine, methotrimeprazine and 4670 RP, which were more effective against histamine than against 5-hydroxytryptamine. Doses as low as 0.1 mg./kg. of methotrimeprazine prevented the oedema produced by histamine, eggwhite, dextran and compound 48/80, and this new derivative of phenothiazine is the most active antagonist of the locally-induced anaphylactoid reaction that we have so far tested. The approximate effectiveness of these derivatives in inhibiting the oedema responses is shown in Table IV, where the activity of the most potent (methotrimeprazine) is taken as 100%. It is of interest that with the more potent

TABLE III

THE EFFECTS OF INTRAVENOUS DOSES OF DERIVATIVES OF PHENOTHIAZINE ON THE LOCAL OEDEMA REACTION PRODUCED IN RATS BY 5-HYDROXYTRYPTAMINE (5-HT), HISTAMINE, DEXTRAN, EGGWHITE AND COMPOUND 48/80

Responses measured in terms of the maximal (100%) found in saline-treated rats.

Derivative						
Name or No.	Dose (mg./kg.)	5-HT	Histamine	Dextran	Eggwhite	Compound 48/80
Promethazine (3277 RP)	2	100	40	32	75	75
	4	60	33	29	28	29
	8	16	16	0	0	12
Chlorpromazine (4560 RP)	0·5	60	60	22	22	90
	1	30	60	0	0	55
	2	30	30	0	0	60
	4	15	15	0	0	20
Methylpromazine (4627 RP)	0·5	92	42	25	29	21
	1	66	12	4	12	25
	2	34	8	8	1?	4
	4	20	2	10	10	0
4670 RP	0·5	83	33	29	28	68
	1	67	17	5	15	25
	2	17	0	2	4	17
Trimeprazine (6549 RP)	0·25	100	14	50	41	59
	0·5	50	18	25	17	25
	1	54	2	4	0	4
Methotrimeprazine (7044 RP)	0·1	62	16	19	23	23
	0·25	25	8	4	4	6
	1	0	5	0	0	0

TABLE IV

APPROXIMATE EFFECTIVENESS OF SOME DERIVATIVES OF PHENOTHIAZINE IN INHIBITING THE OEDEMA PRODUCTION BY 5-HYDROXYTRYPTAMINE (5-HT), HISTAMINE, DEXTRAN, EGGWHITE AND COMPOUND 48/80

Activity of methotrimeprazine is taken as 100%.

Compound	5-HT	Histamine	Dextran	Eggwhite	Compound 48/80
Promethazine	8	2	2	2	2
Chlorpromazine ..	16	3	20	20	3
Methylpromazine ..	12	10	10	10	10
4670 RP	25	10	10	10	10
Trimeprazine	25	30	20	20	20
Methotrimeprazine ..	100	100	100	100	100

compounds in this series blueing of the foot was usually easier to inhibit than was swelling.

On the isolated guinea-pig ileum, methotrimeprazine exerted its antihistamine and anti-5-hydroxytryptamine action in concentrations as low as 0.1 ng./ml. One hundred times this concentration did not alter the acetylcholine response (Fig. 2). The 5-hydroxytryptamine response recovered slightly quicker than the histamine response when methotrimeprazine was washed out (Fig. 3).

FIG. 2.—Action of acetylcholine (A, 0·1 μg.), histamine (H, 0·1 μg.) and 5-hydroxytryptamine (HT, 1 μg.) on the guinea-pig ileum. Bath vol., 15 ml. Time in min. From 12·10 to 12.40 p.m., methotrimeprazine (0·1 ng./ml.) was in the bath ; from 1.20 p.m., this concentration was increased to 10 ng./ml.

Inhibition of the Formaldehyde Reaction.—In a few of the experiments of the present series, the inhibitory action of the antagonists was tested against the oedema reaction produced by the local injection of 4 mg. formaldehyde into the dorsum of the foot. This reaction is known to be different from that of the other oedema-producing agents, since swelling and blueing are much more diffuse and spread well above the knee. Further, the reaction occurs when the tissue histamine or 5-hydroxytryptamine has been depleted, and it is unaffected by pretreatment with BOL 148 (Parratt and West, 1957).

Both adrenaline (4 mg./kg.) and noradrenaline (10 mg./kg.) prevented the swelling and blueing produced by injections of formaldehyde. However, it was only a temporary inhibition, since the response had become maximal when measurements were again made 4 hr. after injection. The most active of the phenothiazine derivatives in preventing the formaldehyde reaction was methotrimeprazine, which was effective in doses of 1 mg./kg. Again the inhibition was of a temporary nature, the formaldehyde response being maximal about 4 hr. after injection.

DISCUSSION

The results of the present experiments using the specific anti-5-hydroxytryptamine substance, BOL 148, add further support to the hypothesis that 5-hydroxytryptamine plays a major rôle in the anaphylactoid reaction in the rat. Small doses are effective in preventing the oedema reaction of egg-white, dextran, compound 48/80 and 5-hydroxy-

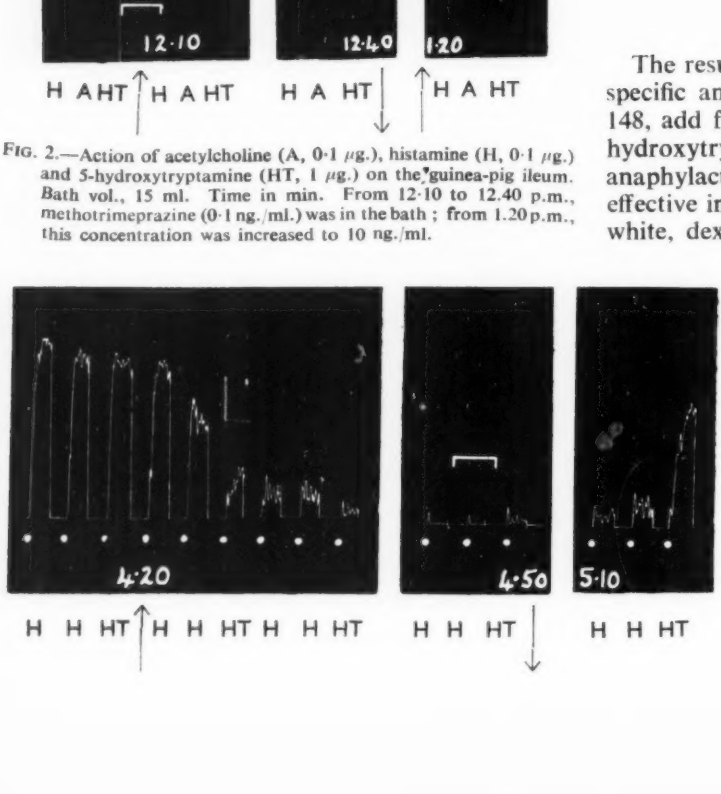

FIG. 3.—Action of histamine (H, 0·1 μg.) and 5-hydroxytryptamine (HT, 1 μg.) on the guinea-pig ileum. Bath vol., 15 ml. Time in min. From 4.20 to 4.50 p.m., methotrimeprazine (1 ng./ml.) in the bath.

tryptamine, leaving that of histamine unchanged. On the other hand, larger doses of the antihistamine substances are generally required and even then the relative effectiveness of such compounds appears to depend not so much on their antihistamine potency as on their ability to antagonize the actions of 5-hydroxytryptamine. Thus promethazine is much more effective than mepyramine in preventing the anaphylactoid reaction since the former possesses greater anti-5-hydroxytryptamine activity than does the latter. Phenindamine and thenalidine likewise are superior to mepyramine in this respect. Halpern and Briot (1950) have screened many such antihistamine substances for their effectiveness in reducing the oedema induced in rats by injections of eggwhite.

The results of the experiments using the two antimetabolites of 5-hydroxytryptamine were disappointing. Most of this type of substance are active *in vitro* against 5-hydroxytryptamine but weakly active in living animals. However, the two used in the present experiments are water-soluble and active orally when given to dogs (Shaw and Woolley, 1956). Yet intravenous doses were virtually devoid of any anti-5-hydroxytryptamine action in the oedema reaction and did not markedly influence the local response of any of the other agents. It is possible that the conditions used in the present experiments were not optimal for the antagonistic action and further work along these lines is in progress.

Much work has already been carried out with sympathomimetic amines and the anaphylactoid reaction in rats. For example, Clark and MacKay (1949) showed that adrenaline, noradrenaline and isoprenaline were capable of modifying the eggwhite response, though the relative effectiveness of these compounds varied greatly. In the present experiments, vasoconstriction does not appear to be the chief reason for the inhibition of the responses by adrenaline since noradrenaline, a more potent vasoconstrictor amine, is much less active. A metabolic action of adrenaline may thus be involved, though we have as yet no proof of this.

The phenothiazine derivatives, first studied for their chemotherapeutic properties, were later shown to possess antihistamine properties. Further work indicated that they were potent substances in the protection of rats against dextran oedema, a property which was linked until very recently with their antihistamine potency. It is now certain that the anti-5-hydroxytryptamine potency of a compound is also important when considering inhibition of the anaphylactoid reaction. Methotrimeprazine is the most potent inhibitor we have used as yet, and experiments have shown that it is also active *in vitro* against both histamine and 5-hydroxytryptamine in extremely low concentrations. Chlorpromazine is about one-fifth as active as methotrimeprazine in inhibiting the oedema induced by local injections of dextran or eggwhite and its anti-5-hydroxytryptamine and antihistamine properties are likewise weaker. It is clear from Table IV that an increase in the length of the side chain in this series of compounds results in increased activity against oedema production (compare promethazine with chlorpromazine), and further any substitution in the side chain also increases activity (compare trimeprazine with methylpromazine).

Measurement of the oedema reaction has involved an assessment of both the swelling and blueing of the foot. Visual records have been made and the results averaged. Usually oedema and blueing go hand in hand, though the mechanisms involved may be different. Gözsy and Kato (1956), for example, have suggested that blueing of the foot is proportional to the phagocytic power of the endothelial cells of the blood vessels, whereas swelling is simply the increase in tissue permeability. Thus the two criteria may be quite separate, and the more active phenothiazine derivatives have occasionally been found to prevent the blueing, but not the swelling, of the foot when histamine or 5-hydroxytryptamine has been injected locally. More recently, Sparrow and Wilhelm (1957) have shown that mepyramine reduces the intensity of colour, but not the area of blueing, of the lesions induced by compound 48/80 or by 5-hydroxytryptamine.

The inhibition of the oedema reaction in the hind-paw of the rat can be used as a useful screening test for new antagonists of 5-hydroxytryptamine and histamine. The drug can be given intravenously 30 min. before the local injections of 5-hydroxytryptamine, histamine, eggwhite or dextran and the responses measured 1 to 2 hr. later. It is possible that this test is more useful for screening new compounds than is the *in vitro* test using antagonism on the guinea-pig ileum.

We wish to thank Upjohn Laboratories, Kalamazoo, for the gifts of 5-hydroxytryptamine creatinine sulphate; Burroughs Wellcome Ltd., London, for compound 48/80; Sandoz Ltd., London, for thenalidine (Sandosten) and BOL 148; Roche Products, Ltd., Welwyn Garden City, for phenindamine (Thephorin); Dr. E. Shaw for the antimetabolites of 5-hydroxytryptamine; and Dr. P. Viaud for the phenothiazine derivatives.

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THE ACTION OF ATROPINE ON PANCREATIC SECRETION

BY

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The action of atropine, in preventing pancreatic secretion in response to a parasympathomimetic drug, is analysed. Atropine does not appear to affect the uptake of glycine by the pancreatic cell, or the incorporation of radioactive amino acids into the total pancreatic tissue proteins, or into the proteins of the zymogen granules. The rate of amylase resynthesis in stimulated glands is not affected by atropine. It is suggested that atropine blocks pancreatic secretion in rats by blocking the extrusion of zymogen granules.

Although the action of atropine in inhibiting cell secretion has been the subject of several papers (for a review see Henderson and Sweeten, 1943), we could find no references to experimental studies on the mechanism of action of this drug. We present here the results of a series of experiments undertaken in order to analyse this problem.

The net effect of atropine on the pancreas is to block the secretion elicited by parasympathomimetic drugs. Cell secretion is a complex phenomenon where one can distinguish at least three distinct phases: (1) *ingestion*, during which the raw material penetrates the cell; (2) *synthesis*, consisting of the building up of the secretion product; and (3) *extrusion*, a process in which this material is extruded from the cell (for a detailed analysis of cell secretion see Junqueira and Hirsch, 1956). Atropine might act on any one of these stages, or on more than one, to block secretion.

To analyse the action of atropine on ingestion, the rate of penetration of a radioactive amino acid into the pancreatic cell and its proteins was studied in control and atropine-treated animals. If atropine blocks ingestion, a decrease in the rate of penetration would be expected.

To study the action of atropine on the phase of synthesis the following estimations were made in atropine-treated and control animals: (a) the rate of incorporation of amino acids into the total pancreatic proteins; (b) the rate of incorporation of amino acids into the zymogen granule proteins; and (c) the rate of resynthesis of a pancreatic digestive enzyme after its depletion by parasympathomimetic stimulation. If atropine acts by inhibiting the synthetic processes in the cells, a decrease of the rate of amino-acid incorporation into the total proteins and zymogen granule proteins, and also of the rate of resynthesis of the enzyme, would be expected.

If, however, none of the above mechanisms operate one would be led, by exclusion, to postulate that atropine probably acts mainly on the phase of extrusion. This, indeed, is suggested by the results presented in this paper.

MATERIAL AND METHODS

Adult male Wistar rats were used throughout. To study the rate of penetration of an amino acid into the rat pancreatic cell, ether-anaesthetized animals (200 to 250 g.) were stimulated by subcutaneous injection of a 2.5% solution of methacholine bromide (acetyl-β-methylcholine bromide, 30 mg./kg.). After 5 min., a 10% atropine sulphate solution (100 mg./kg.) was administered, also subcutaneously, followed 3 min. later by an intravenous injection of glycine-1-[^{14}C] (10 μc./100 g.) in saline. In this experiment one group of animals (5) received only saline; the second (6) methacholine and saline, and the third (5) methacholine and atropine. Pancreatic biopsies were taken 2, 5, 15, and 30 min. after the glycine injection. Total and protein-bound radioactivity were assayed in 25 λ aliquots of 8% aqueous homogenates of the biopsy material according to the technique described by Junqueira, Hirsch, and Rothschild (1955) and Rothschild, Hirsch, and Junqueira (1957), with thin mica-window Geiger tubes (TGC 2 of Tracerlab, Boston, U.S.A., with 1.7 mg./sq. cm.).

To study the rate of incorporation of phenylalanine and glycine into the proteins of the rat pancreas, and into the zymogen granule proteins, rats (250 to 300 g.) were anaesthetized and their pancreases stimulated as mentioned above. After 55 min. a 10% atropine sulphate solution (100 mg./kg.) was administered subcutaneously, followed 5 min. later by an intravenous injection of 7 μc./100 g. of DL-phenylalanine-3-[^{14}C] or 5 μc./100 g. of glycine-1-[^{14}C] in saline. In the control group the animals were treated likewise.

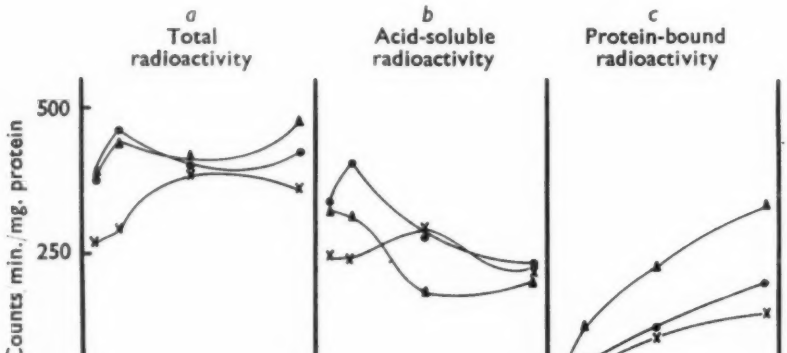

FIG. 1.—Penetration of glycine-1-[^{14}C] into the pancreatic cell. | ●——●, control; X——X, methacholine; ▲——▲, methacholine and atropine. In the methacholine experiment each point is the average of 6 rats, whereas for the control and atropine experiments 5 animals were used in each group. Note that the total radioactivity shows no definite trend to increase or decrease, and that, while the acid-soluble radioactivity decreases, the protein-bound radioactivity increases.

but received saline injections instead of atropine. Five and three animals in each group were used for the glycine and phenylalanine experiments respectively. Pancreatic biopsies were performed under ether anaesthesia 30, 150, and 540 min. after giving the radioactive amino acid. The biopsy material was homogenized in 0.25 M-sucrose and the zymogen granules isolated by the technique of Hokin (1955). Measurements of protein-bound radioactivity of the total pancreatic proteins, as well as of the zymogen granules, were performed as described above.

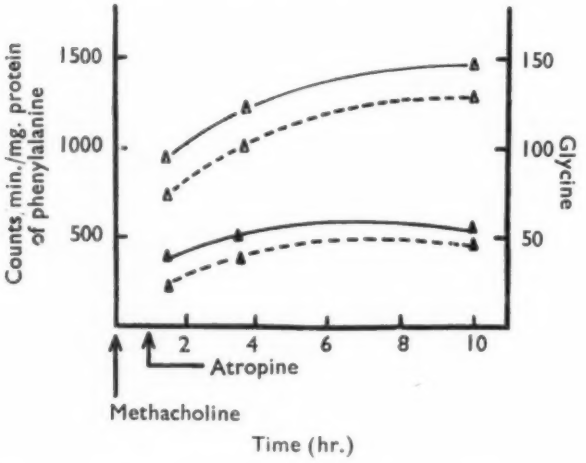

FIG. 2.—Incorporation of phenylalanine-3-[^{14}C] and glycine-1-[^{14}C] into the pancreatic proteins. Full lines are control experiments while the broken lines denote atropine experiments. The open triangles refer to glycine and the closed triangles to phenylalanine. In the phenylalanine experiment each point is the average of 3 rats, while in the glycine experiment 5 animals were used for each group. These curves suggest that atropine does not influence the incorporation rate.

Amylolytic activity was assayed in 200-fold diluted 1% aqueous homogenates of the pancreatic biopsy material of animals treated as described for the incorporation experiments by the method of Smith and Roe (1949).

To visualize the entrance of radioactive glycine into the pancreatic cells, autoradiography was performed on 4 μ thick histological sections fixed with 10% formol solution in M/150 phosphate buffer at *p*H 7.2. The stripping film technique described by Doniach and Pelc (1950) was followed, using Kodak Scientific Plates emulsion and an exposure time of 75 days.

The results of all experiments were analysed by Student's *t* test.

RESULTS

The results obtained on the penetration of glycine into the pancreatic cells—that is, on the ingestion phase—are shown in Fig. 1. The rate of penetration follows a similar curve in the 3 groups of animals studied (Fig. 1*a*). Therefore, it seems that atropine does not act on cell secretion by depressing the uptake of the amino acid used. Furthermore, Fig. 1*b* shows that the decrease of

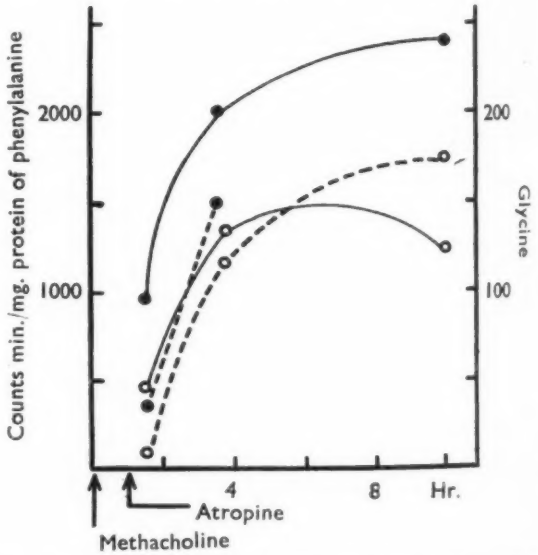

FIG. 3.—Incorporation of phenylalanine-3-[^{14}C] and glycine-1-[^{14}C] into the proteins of pancreatic zymogen granules. The number of animals used is the same as in Fig. 2. Atropine does not influence the incorporation rate. ○——○, control; ○ - - - ○, atropine; ●——●, phenylalanine; ● – – ●, glycine.

acid-soluble radioactivity in this material is not different for the various groups. The protein-bound radioactivity (Fig. 1*c*) in the atropine-treated animals is, however, significantly ($P<0.05$) higher than in the methacholine-treated group; but no plausible explanation can be given at present for this.

Negative results were also obtained when studying the influence of atropine on the synthesis of the zymogen granule and total pancreatic proteins. Figs. 2 and 3 show similar curves for the incorporation rates of phenylalanine and glycine in both atropine-treated animals and their controls.

As for the results obtained on amylolytic activity, it is obvious (Fig. 4) that atropine did not decrease the rate of amylase resynthesis in the treated animals when compared with their controls.

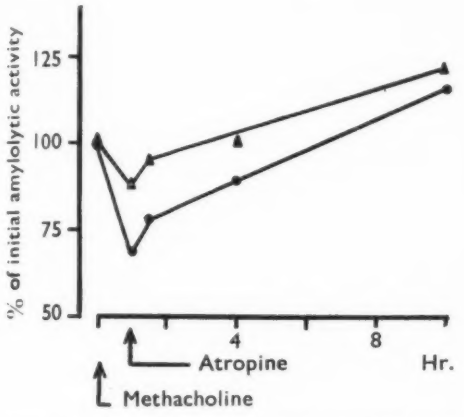

FIG. 4.—Amylolytic activity in successive pancreatic biopsies after parasympathomimetic stimulation followed by saline (●) or atropine (▲) injection. Each point is the average of at least 7 animals. Atropine does not affect significantly the rate of amylase resynthesis (Student's *t* test).

The results of these experiments showed no statistically significant difference between the control and atropine-treated groups.

From the examination of the autoradiographs of the pancreas of the atropinized animals and their controls, it was impossible to distinguish one group from the other. Thus, under the conditions of our experiment, the administration of atropine did not inhibit the penetration of glycine into the cells of the pancreas. The intensity of the autoradiographs varied from animal to animal, irrespective of whether atropine had been given or not.

Our results suggest, therefore, that the main action of atropine on the rat pancreatic exocrine secretion is to block the extrusion of zymogen granules elicited by parasympathomimetic stimulation.

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SOME EFFECTS OF HISTAMINE IN THE NORMAL AND *HAEMOPHILUS PERTUSSIS* VACCINATED RAT

BY

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The effects of histamine in the normal and *H. pertussis* vaccinated rat have been investigated. In the normal animal small doses of histamine (from 1 to 30 mg./kg., i.v.) produced significant, reversible alterations in the blood pressure, electrocardiogram and electroencephalogram. Doses of 100 mg./kg. or more caused bronchoconstriction and doses of 200 mg./kg. or more an effect on the muscular contractions after electrical stimulation of the nerve. Acute histamine intoxication resulted on rapid injection of 500 mg./kg. intravenously; death was probably due to cardiovascular collapse. In the vaccinated rat, a distinction could be made between the immediate and delayed effects of histamine. With a sublethal dose of histamine (5 mg./kg.) alterations in the blood pressure, electrocardiogram and electroencephalogram were noted. The neuromuscular system, which in the normal animal was the least reactive to histamine, showed the most marked increase in sensitivity in the vaccinated rat. The delayed effects of histamine observed after an interval of 5 to 30 min. were characteristic and irreversible, leading to death of the animal. The mechanism of death in the vaccinated rat after histamine appears to differ from that of the normal animal and has been discussed in the light of present knowledge.

The ability of the rat and mouse to tolerate large doses of histamine is well known, and stands in sharp contrast to the high sensitivity of other laboratory animals such as the guinea-pig, rabbit, or cat, which succumb when relatively small doses of this substance are injected (Guggenheim, 1940 : Bovet and Bovet-Nitti, 1948). Little is known of the mechanism underlying death of the rat and mouse due to histamine intoxication or the reason for this high natural resistance. The induction of anaphylactic sensitivity is more difficult in histamine resistant than in histamine sensitive species (Mayer and Brousseau, 1946 ; Perry and Darsie, 1946). Antihistamines protect the guinea-pig, rabbit or dog from many lethal doses of histamine but do not protect the mouse against a single lethal dose (Bovet and Walthert, 1944 ; Halpern and Wood, 1950).

A number of investigators have shown that it is possible by adrenalectomy to render the rat and mouse considerably more sensitive to the action of histamine (Wyman, 1928 ; Halpern and Wood, 1950 ; Houssay, 1955). In the adrenalectomized mouse, adrenaline and cortisone increase resistance to histamine, and when both are injected the normal resistance to histamine is restored (Halpern, Benacerraf, and Briot, 1952). Houssay (1955) has shown that the sensitivity of adrenalectomized rats to histamine is largely due to the loss of cortical function.

A further method of increasing the sensitivity of mice to histamine was first described by Parfentjev and Goodline (1948). They made the interesting observation that mice injected with *H. pertussis* vaccine developed a considerable sensitivity to histamine which reached its maximum 5 days after vaccination. This phenomenon has been confirmed by a number of workers (Halpern and Roux, 1950 ; Kind and Wood, 1953 ; Maitland, Kohn, and Macdonald, 1955). Experiments carried out with the rat showed that this animal could similarly be rendered more sensitive to histamine after *H. pertussis* vaccine. The guinea-pig failed to become more histamine-sensitive when thus treated (Maitland, Kohn, and Macdonald, 1955). Antihistamines when given before a challenge dose of histamine to the vaccinated animal were effective in preventing the onset of the fatal syndrome (Parfentjev and Goodline, 1948 ; Halpern and Roux, 1949 ; Thiele and Schuchardt, 1952). A number of possible explanations have been advanced to account for the effect

of the vaccine. It may exert an injurious effect on the adrenals and render the animal more sensitive to histamine, since protection is obtained with cortisone (Kind, 1953). Mouse lung tissue is normally rich in histaminase, and it has been shown that the vaccinated lung tissue detoxifies considerably less histamine (Kind and Wood, 1953). However, histological examinations of the vaccinated rat failed to reveal pathological changes in the adrenals (Malkiel and Hargis, 1952). Thiele and Schuchardt (1952) demonstrated that histamine sensitization by *H. pertussis* vaccine could be inhibited by repeated treatment with quercitin during the sensitizing period. This substance is known to prolong the action of adrenaline and decrease cutaneous capillary permeability.

To obtain further information on the action of histamine in the normal and *H. pertussis* vaccinated rat, experiments were designed to study such action on the cardiovascular system, smooth muscle, the central nervous system and neuromuscular transmission.

Materials and Methods

The experiments were carried out on normal and vaccinated rats of both sexes weighing 200 to 400 g. An *H. pertussis* vaccine (BV) containing 200,000 million organisms/ml. and resuspended in saline was used. Animals were injected intraperitoneally with 80,000 million organisms/100 g. of body weight. This standard dose of organisms was employed as it has been shown to sensitize appreciably to histamine (Maitland *et al.*, 1955). The height of histamine sensitization was reached 4 days after vaccination and remained at this level for approximately 2 weeks, hence all tests with the treated animals were undertaken during this period of maximum sensitivity. Histamine in the form of the dihydrochloride was used and all doses are stated in terms of this salt. Unless otherwise indicated the animals were under pentobarbitone sodium anaesthesia (40 mg./kg., i.p.) and in some experiments gallamine was given (10 mg./kg., i.v.). All intravenous injections were made into the tail vein of the rat.

Blood Pressure.—The common carotid artery was cannulated and the blood pressure was recorded in some experiments with a mercury manometer and in others by means of an electromanometer (Frank and Longo, 1955). With the latter system large movements of the anticoagulant in the tube during pressure variations were avoided. To reduce coagulation a small dose of heparin was injected.

Electrocardiogram.—The electrical potentials were recorded from the two fore feet and the left hind foot by insertion of 3 needle electrodes into the skin of the animal. The same recording apparatus was used as for the electroencephalographic recordings.

Tidal Air.—The method of Konzett and Rössler (1940) was employed, based on determination of the tidal air maintaining the volume and pressure of ventilation constant.

Cerebral Electrical Activity.—Four stainless steel electrodes were screwed through the skull overlying the frontal and parietal lobes and the cerebral electrical activity was recorded by the bipolar method. In some experiments the fixation of the electrodes was carried out on animals given light ether anaesthesia, immobilized by the intravenous administration of gallamine and maintained under artificial respiration (30 strokes/min.) with air. The recordings were started 1 hr. after completing these preparations. The electrical activity of the brain was recorded by means of a 4 channel Grass Electroencephalograph (Model III D).

Electrical Stimulation of the Masseter Muscle.—The technique described by Eicholtz, Hotovy, and Erdniss (1949) and modified by Marotta (1957) was employed. The rat, after tracheotomy, was maintained under artificial respiration. The animal was fixed on a wooden board, and the upper jaw held up by means of a spring placed under the upper incisors. A rod connected to a writing lever was attached to the lower incisors. The lower jaw was held down by the weight of the rod. Two electrodes were inserted into the left masseter muscle, which was stimulated once every min. At each stimulation the writing lever recorded the movement of the lower jaw on a slowly moving smoked drum.

Gastrocnemius-sciatic Nerve Preparation.—The contractions of the gastrocnemius muscle following electrical stimulation of the cut peripheral end of the sciatic nerve were recorded isometrically. In some experiments contractions of the gastrocnemius and masseter muscles were recorded simultaneously in the same animal.

Phrenic Nerve-diaphragm Preparation.—The technique described by Bülbring (1946) was employed, as modified by Bovet-Nitti (personal communication). The bath was maintained at 30°, and the nerve stimulated every 30 sec. A shock of 2 to 3 V. was applied for approximately 1 m.sec. The solution employed was that described by Taugner and Fleckenstein (1950). The drug under examination was added to the bath of 150 ml. capacity and allowed to remain for 15 min. Traces of the drug were removed by washing 3 times, and the muscle was left to recover for 15 min. before a fresh test was undertaken. Electrical stimulation of the nerve was continued during the recovery period.

Results

Normal unanaesthetized rats when injected with doses of histamine ranging from 30 to 300 mg./kg. intravenously reacted by manifestations of pain and subsequent shock. They sat in a crouched position and breathed with difficulty. Copious salivation, urination, defaecation, cyanosis, and paralysis of the extremities were also observed. When given by rapid intravenous injection 500 mg./kg. or more of histamine caused the imme-

diate death of the animal in convulsions. The intraperitoneal injection of 2.5 to 3 g./kg. of histamine resulted ultimately in death of the animal, but this was noted to occur from 5 min. to several hr. after the injection, with symptoms similar to those described above. The animal usually died in convulsions. No significant protective effect towards histamine intoxication was afforded by anaesthesia.

With the dose of vaccine injected, more than 90% of the animals could be sensitized. Although it was usually possible to kill the sensitized rat with 10 mg./kg. histamine (i.v.), this dose was considered too near to that from which the animal survived (5 mg./kg., i.v.). Hence in most of the sensitization experiments, doses of 20 mg./kg. histamine (i.v.) were used because this proved invariably fatal. When this dose was injected into the vaccinated rat, symptoms identical to those described for the normal animal were observed. However, whereas the normal rat after a certain time completely recovered, the conditions of the vaccinated animal progressively deteriorated until it died. Death occurred from 5 min. to several hours after the injection. No difference in the speed which which the animal died was observed whether histamine was given intravenously or intraperitoneally.

It was clear that, whereas a lethal dose of histamine given intravenously in the normal animal provoked instantaneous death, the smaller lethal dose of histamine in the vaccinated rat, even when given intravenously, required a minimum period of time, which in no instance was less than 5 min., before the animal succumbed.

Blood Pressure.—The average blood pressure in the normal anaesthetized rat was 140 to 160 mm. Hg. Following the intravenous administration of 1 mg./kg. histamine in the normal rat, a significant lowering of the blood pressure was noted (to 80 to 90 mm. Hg), which then slowly rose to its original level. Large doses of histamine (300 mg./kg.) provoked a pronounced lowering in the blood pressure (to 40 to 50 mm. Hg), which remained low for more than an hour. It was generally observed that with small doses of histamine a hypertension of very short duration preceded the subsequent hypotension. The blood pressure of the vaccinated rat was similar to that of the normal animal and the immediate effects of histamine were similar in both cases. However, the administration of not less than 20 mg./kg. of histamine (i.v.) provoked an arterial hypotension which tended to progress until the death of the animal. In two experiments with vaccinated rats, it was noted that 2 min. after the administration of 50 mg./kg. histamine (i.v.) the arterial blood pressure after the usual fall rose slowly. At this point, simultaneously with the appearance of grave alterations in the electrocardiogram, a sudden fall in the blood pressure ensued, and death followed shortly afterwards.

Electrocardiogram. — The electrocardiogram (ECG) was recorded in the rat under pentobarbitone sodium anaesthesia. It was not possible to obtain a satisfactory tracing for the unanaesthetized rat owing to the anxiety and fear of the animal, which led to an increased frequency in the cardiac rhythm. A large number of control experiments were carried out to determine the reproducibility of the tracings, and it was found that the average heart rate varied roughly from 300 to 450/min. depending to some extent on the age of the animal. These figures agree with the findings of Heise and Kimbel (1955). Daily ECG records of the same animal were taken for a period of five days ; these were remarkably constant, only minor modifications being noted.

In the normal animal, doses of histamine up to 200 mg./kg. (i.v.) did not produce any significant change in heart rate. With the slow injection of larger doses (500 mg./kg.) there was a significant reduction in the heart rate within 1 min. of the injection. Thus in a typical experiment the frequency was reduced from 342 to 198/min. A rapid return took place and within 2 min. the rate was 282/min. Changes in the form of the ECG were observed when small doses of histamine (10 mg./kg., i.v.) were administered, and consisted of modifications of the S-T segment and enlargement of the T wave (Fig. 1A). A complete return to normal was obtained. With larger doses of histamine (200 mg./kg., i.v.) a complete and reversible change in the ECG was found (Fig. 1B). The rapid intravenous injection of 500 mg./kg. histamine (lethal dose) caused the immediate flattening and failure of the ECG. The intraperitoneal injection of 2.5 g./kg. histamine was followed by a reduction in heart rate and voltage. In some cases arrhythmia was noted (Fig. 1C).

In the vaccinated animal a sublethal dose of histamine (5 mg./kg.) produced some changes in the heart rate, consisting in a slight preliminary rise, followed by a fall (Fig. 9*a*, upper diagram). With 20 mg./kg. histamine (lethal dose) a slight increase in the number of beats was first noted (300 to 318/min.) which may have been related to the brief period of hypertension. The frequency then began to decrease gradually (Fig. 9*b*, upper diagram). With 100 and 200 mg./kg. a fall in the frequency was obtained within the first min. of

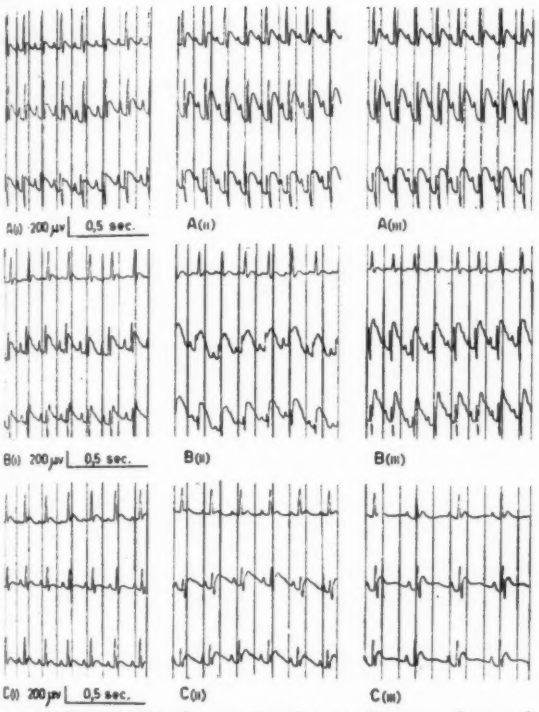

FIG. 1.—Effect of histamine on the electrocardiogram of normal rats anaesthetized with pentobarbitone sodium (40 mg./kg., i.p.). A (I), ECG of a normal rat weighing 230 g.; A (II), 1 min. after 10 mg./kg. histamine (i.v.); and A (III) 8 min. after histamine. B (I), ECG of a normal rat weighing 200 g.; B (II), 4 min. after 200 mg./kg. histamine (i.v.); and B (III), 90 min. after histamine. C (I), ECG of a normal rat weighing 190 g.; C (II), 20 min. after 1·5 g./kg. histamine (i.p.); and C (III), 45 min. after C (II) and 12 min. after a lethal dose of histamine (2·5 g./kg., i.p.).

the injection, which then partly recovered to fall again later (Fig. 9*c*, upper diagram). Fig. 2 (D and E) shows the ECG changes which occurred on injecting histamine in the vaccinated animal. An almost complete arrhythmia and extra-systoles developed and the animal did not recover.

A further series of records were taken from a group of rats which were given daily doses of 50 mg./kg. histamine (i.v.) before vaccination and subsequently during the sensitization period for a total of 5 days. The object of these experiments was to determine the time at which changes in the ECG could be noted. During the first 2 days after vaccination no significant changes occurred (that is, the animals had not become sensitized to histamine) whereas from the 3rd to the 5th day death occurred when the histamine was injected. Rats treated daily with 50 mg./kg. histamine did not become desensitized to the lethal effect of histamine when challenged after the establishment of the hypersensitivity.

Tidal Air.—Registration of the tidal air was selected to test the reactivity of histamine on

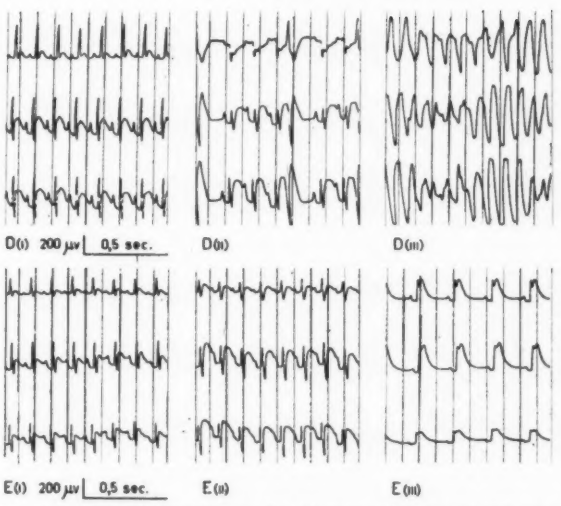

FIG. 2.—Effect of histamine on the electrocardiogram of rats vaccinated with *H. pertussis*. The animals were anaesthetized with pentobarbitone sodium (40 mg./kg., i.p.). D (I), ECG of vaccinated rat weighing 200 g.; D (II), 5 min. after 50 mg./kg. histamine (i.v.); and D (III), 8 min. after histamine. E (I), ECG of vaccinated rat weighing 220 g.; E (II), 20 min. after 200 mg./kg. histamine (i.p.); and E (III), 45 min. after histamine.

smooth muscle. In the rat this organ was found to be only slightly sensitive to the action of histamine. With doses of 100 mg./kg. (i.v.) of histamine a reduction in tidal air, during constant pressure ventilation, attributed to bronchospasm was observed, which lasted for only a short period (Fig. 3). Rapid injection of 500 mg./kg. (i.v.) histamine provoked a marked reduction of tidal air which persisted even after death (Fig. 3).

The actions of histamine on the bronchial musculature of the vaccinated rat may be divided into immediate and delayed effects. The immediate action was similar to that observed in the

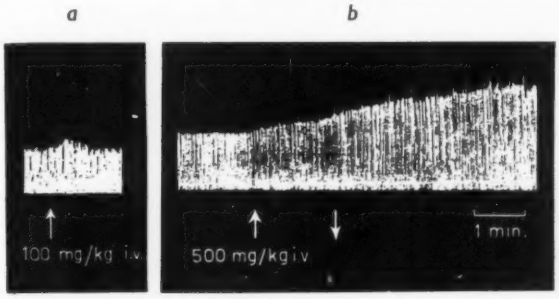

FIG. 3.—Effect of histamine, given intravenously, on the tidal air during constant positive pressure ventilation of the normal rat under pentobarbitone sodium (40 mg./kg., i.p.) and gallamine (10 mg./kg., i.v.). Method of Konzett and Rössler (1940). A rise in the record indicates a fall in tidal air. (*a*) Rat; 250 g. 100 mg./kg. histamine was the smallest dose which produced a decrease in tidal air. (*b*) Rat; 380 g. Rapid administration of 500 mg./kg. histamine. The second arrow indicates cardiac arrest. The decrease in tidal air continued even after death.

normal animal ; the minimum effective dose which produced a distinct bronchospasm was again 100 mg./kg. (i.v.). In some instances this action was of considerable duration. Administration of 500 mg./kg. histamine (i.v.) once again led to death of the animal with a bronchospasm similar to that observed in the normal animal. The delayed effects of histamine were very characteristic. The injection of 50 mg./kg. (i.v.) histamine in the normal rat was without noticeable effects, but a similar dose given to the vaccinated animal produced a bronchospasm after a period of not less than 5 min. There is thus a delayed effect in the vaccinated rat at a dose substantially less than that which affects the normal animal.

Electroencephalogram.—The electroencephalogram (EEG) of the normal rat was similar to that of the vaccinated animal. Under pentobarbitone sodium anaesthesia, the animal showed a cerebral electrical activity characterized by an irregular basal rhythm with a frequency of 8 to 12 c./sec., upon which spike waves, often organized into spindles, were superimposed (Fig. 4A). On this background it was possible to show the desynchronizing effects on application of external stimuli. The administration of further quantities of pentobarbitone sodium provoked a flattening of the tracing, in which the spike potentials became more apparent. The cerebral electrical activity of the curarized unanaesthetized rat was highly desynchronized and consisted of waves with a voltage generally not more than 100 μV. and a frequency of approximately 20 to 30 c./sec. The arousal reaction could not be elicited. In the normal anaesthetized rat the intravenous administration of histamine in doses of 30 to 300 mg./kg. provoked desynchronization similar to that observed after external stimulation (Fig. 4B). This desynchronization tended to be very prolonged and usually lasted longer than the effects of the anaesthetic on the EEG (Fig. 4C). In the normal, curarized rat the administration of histamine (30 to 300 mg./kg.) was followed by a flattening of the cerebral electrical tracing which lasted for 5 to 15 min. In some cases profound modifications in the ECG took place before these changes of the EEG. In other experiments the modifications proceeded in the reverse order. The diminution in frequency and increase in voltage of cerebral potentials in the rat described by Brandon (1955) with the doses of histamine he employed could not be confirmed by the present authors.

The initial effects of histamine were similar in the vaccinated rat. However, whereas in the normal animal a gradual return to normal was observed after sublethal doses, in the vaccinated rat the modifications in the cerebral electrical activity became more pronounced, as shown by a further flattening of the tracings, and finally gave rise to failure (Fig. 5).

The rapid intravenous administration of 500 mg./kg. histamine both in the normal and vaccinated rat was followed by a general flattening of the EEG tracings and death.

Electrical Stimulation of the Masseter Muscle.—Histamine induced paralysis in the rat, and therefore its effect on neuromuscular transmission was examined. In the normal rat the intravenous injection of histamine (100 mg./kg. or more) gave rise in some experiments to an irregular

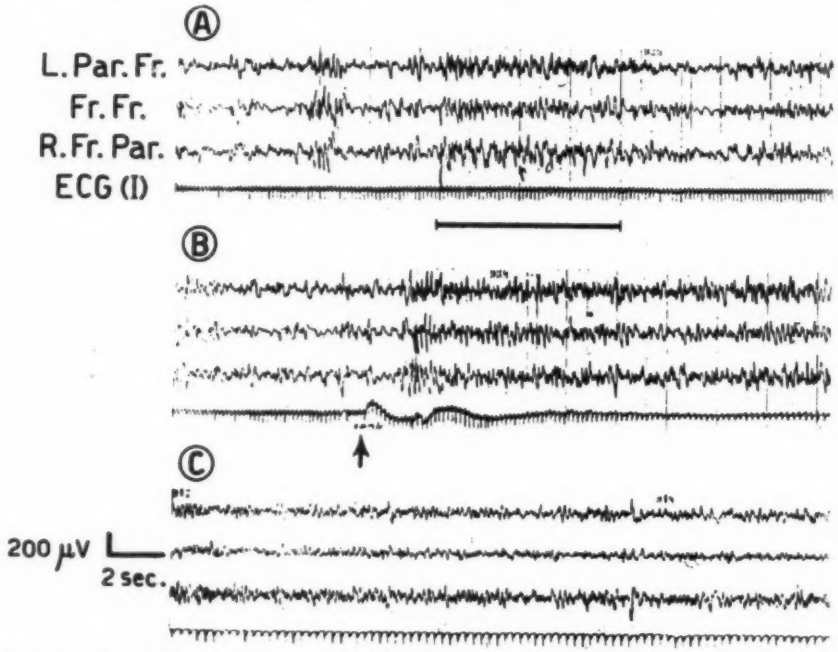

FIG. 4.—Effect of histamine on the electroencephalogram of the normal rat anaesthetized with pentobarbitone sodium (40 mg./kg., i.p.). Leads are as indicated. **(A)** Rat; 380 g. The EEG of normal animal is synchronized with spikes grouped into brief spindles. The arousal reaction (black line) was elicited by pinching of the tail. **(B)** Effect of 150 mg./kg. histamine, i.v. (at arrow). The desynchronization of the EEG is similar to that obtained by means of peripheral stimulation but is more prolonged. **(C)** 15 min. after histamine. The tracing is still desynchronized.

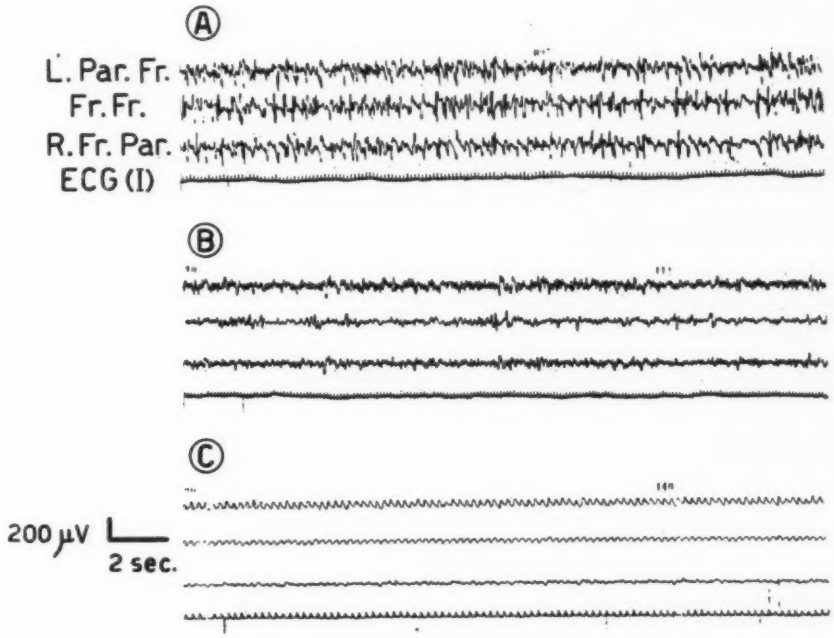

FIG. 5.—Effect of histamine on the electroencephalogram of a rat vaccinated with *H. pertussis*. The animal was anaesthetized with pentobarbitone sodium (40 mg./kg., i.p.). **(A)** EEG of vaccinated animal is synchronized with spikes which are not grouped as spindles. **(B)** 2 min. after 50 mg./kg. histamine, i.v. Desynchronization of the tracing is still present. **(C)** 15 min. after histamine. The tracing is now flattened.

and fairly frequent contracture of the masseter muscle in the absence of electrical stimulation. When a dose of 100 mg./kg. (i.v.) or less of histamine was injected into the normal rat, no effect on the height of the muscular contractions in response to stimuli was noted. With 200 mg./kg. (i.v.) of histamine some effect was sometimes observed (Fig. 6). In two experiments the contractions were 82% and 95% of the original value, and in another two no effect on the muscular contractions was noted. The slow injection of 500 mg./kg. histamine (i.v.) gave rise to a very pronounced reduction in the height of contraction; in four experiments the contractions were reduced to 17, 21, 33, and 94% of the original value (Fig. 6). Generally the maximum degree of paralysis was obtained within 30 min. of injection of histamine and lasted considerably more than 3 hr. Fig. 8 shows the effects of 500 mg./kg. histamine by slow intravenous injection on the heart rate and muscular contractions. It was further observed that a second similar dose of histamine administered after complete recovery produced a far smaller effect on the muscular contractions.

A sublethal dose of histamine in the vaccinated rat (5 mg./kg.) (Fig. 7) produced a significant decrease in the muscular contractions in two experiments (45% and 50% of the original value), but in another two the heights of the contraction were 90% and 100%. In those experiments where a decrease occurred, complete recovery was observed. At the end of the experiments with 5 mg./kg. histamine a challenge dose of 50 mg./kg. histamine was given to establish whether the animals were indeed sensitive. This was confirmed, as all these animals died shortly after injection. With lethal doses of histamine in the vaccinated rat (20 mg./kg., 100 mg./kg., and 200 mg./kg.) a rapid onset in neuromuscular paralysis was observed, leading to complete inhibition of the contraction and death of the animal. It is clear from Fig. 7 that the speed

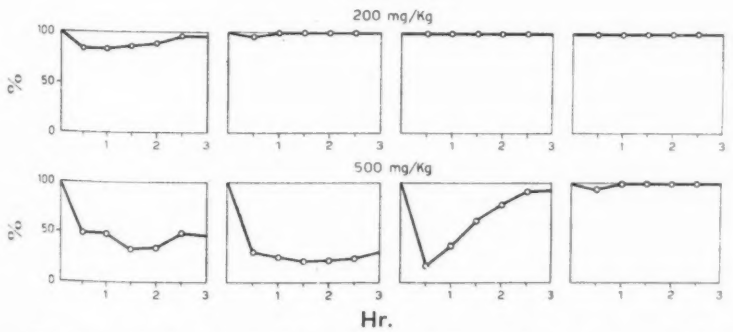

FIG. 6.—The effect of histamine (i.v.) upon the contractions of the masseter muscles (stimulated indirectly) of groups of 4 normal rats. The rats were anaesthetized with pentobarbitone sodium (40 mg./kg., i.p.). Ordinates show % original twitch height.

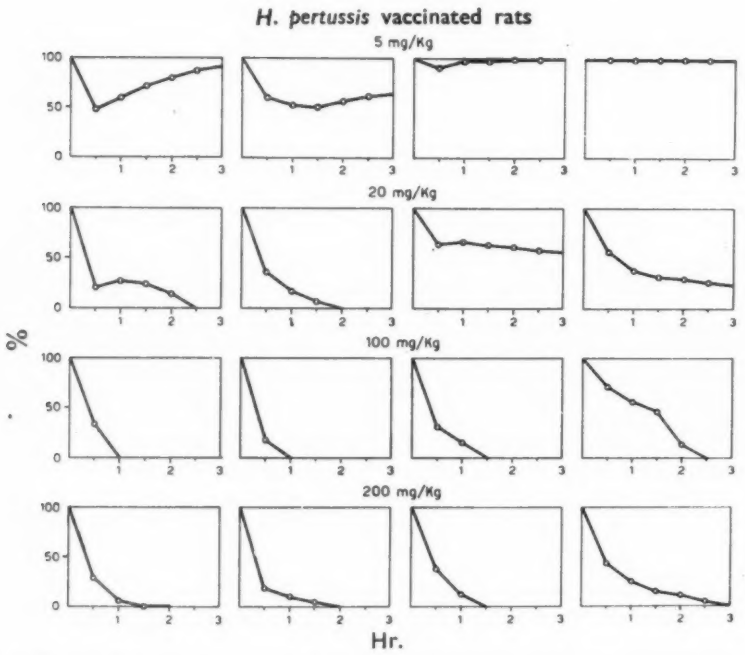

FIG. 7.—The effect of histamine (i.v.) upon the contractions (stimulated indirectly) of the masseter muscles of groups of 4 rats vaccinated with *H. pertussis*. Pentobarbitone sodium (40 mg./kg., i.p.). Ordinates show % original twitch height.

of onset of the neuromuscular paralysis was more pronounced when 100 mg./kg. was given than after 20 mg./kg., but little significant difference was noted between the administration of 100 mg./kg. and 200 mg./kg. histamine. Further, it is of interest to observe from a comparison of Figs. 6 and 7 that the degree of muscular paralysis produced by the injection of 500 mg./kg. histamine in the normal rat was similar to that obtained with 5 mg./kg. in the vaccinated rat. It would thus appear that the vaccinated animal is almost 100 times more sensitive to histamine as judged by this method. Figs. 8 and 9 illustrate the effect of different doses of histamine on the heart rate and muscular contractions of the normal and vaccinated rat. It is clear that there is no relationship between these effects. Thus in the normal animal the intravenous injection of 500 mg./kg. histamine gave rise to a momentary reduction in heart rate which returned to normal and fell again somewhat later, whereas the muscular contractions continued to decrease gradually. After 45 min. when the maximum inhibition of muscular contractions was obtained, the heart rate was still 250/min. (Fig. 8).

In the vaccinated animal, the injection of 5 mg./kg. and 20 mg./kg. of histamine had little effect on the heart rate whereas the muscular contractions were significantly reduced. With 100 mg./kg. and 200 mg./kg. histamine the heart rate very slowly decreased, whereas the inhibition in the muscular contractions was much more rapid (Fig. 9). A close similarity between the sensitivity of the gastrocnemius and masseter muscles to histamine was observed. In most of our experiments we used the masseter.

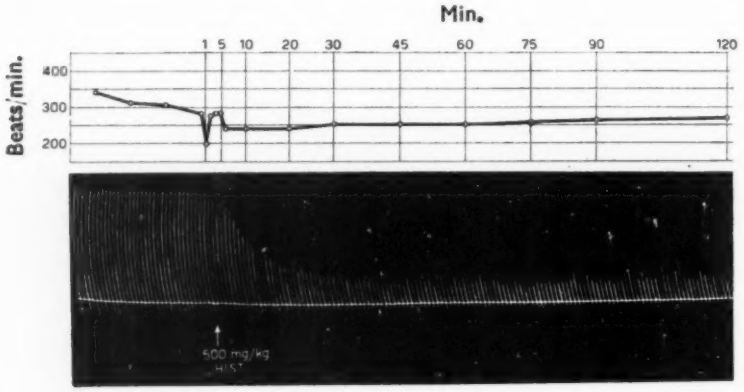

FIG. 8.—Effect of histamine injected intravenously into a normal rat on the heart rate and on the contractions of the masseter muscle stimulated indirectly. Rat (240 g.) anaesthetized with pentobarbitone sodium (40 mg./kg., i.p.).

Phrenic Nerve-diaphragm Preparation.—Various concentrations of histamine were added to the bath containing

nerve-muscle preparations of normal and vaccinated rats. Table I summarizes the results obtained.

It will be noted that the smallest dose of histamine which caused inhibition of the muscular contractions was 10 mg./l. As the dose was increased the degree of paralysis became more pronounced, and with doses of 1 g./l. a paralysis of 40% was obtained. The muscular contractions slowly but usually completely

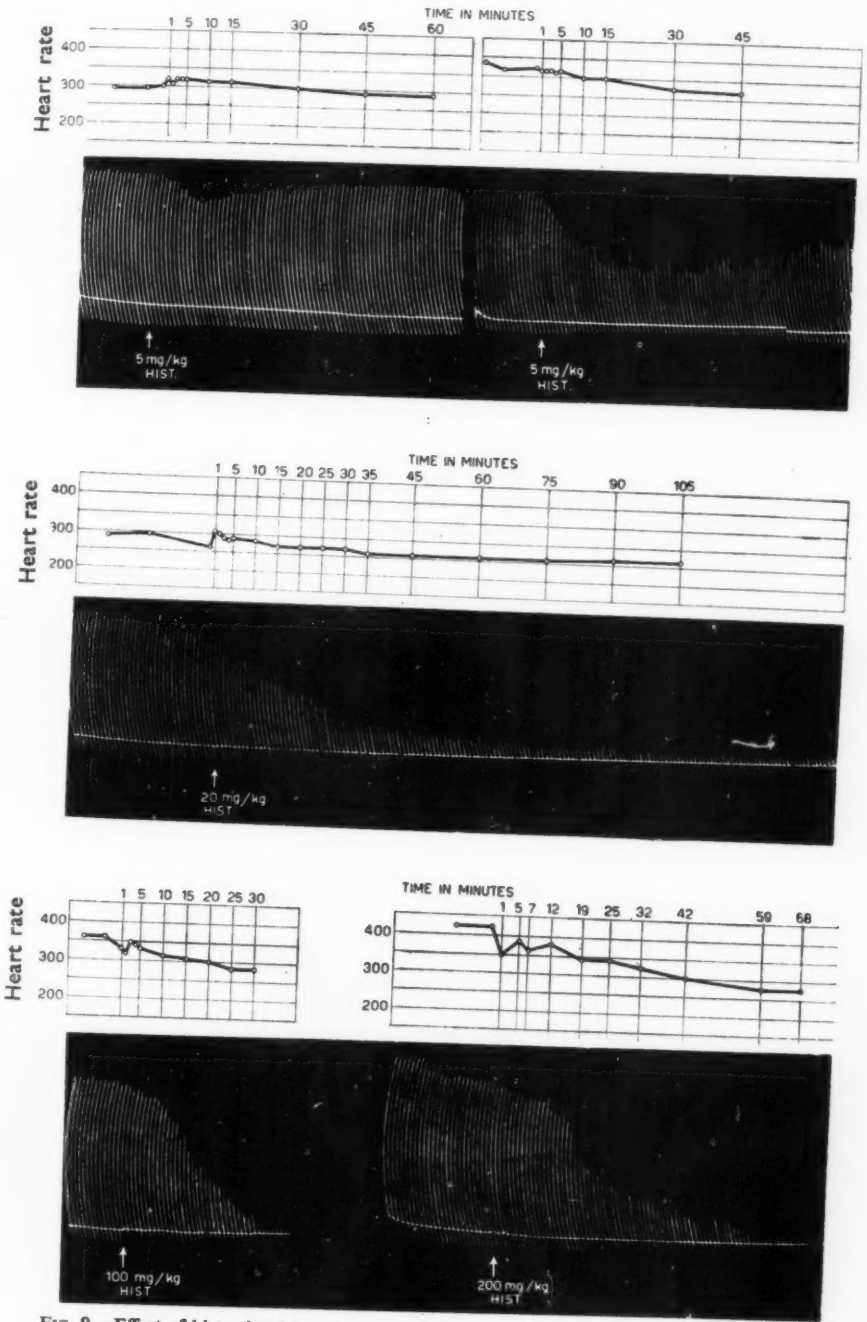

FIG. 9.—Effect of histamine (given intravenously to vaccinated rats) on the heart rate and on the contractions of the masseter muscle stimulated indirectly. Rats anaesthetized with pentobarbitone sodium (40 mg./kg., i.p.).

recovered after repeated washing. It was not possible to show any significant difference between the normal and vaccinated animals by this procedure.

TABLE I

THE EFFECT OF HISTAMINE ON THE PHRENIC NERVE-DIAPHRAGM PREPARATION OF THE NORMAL AND *H. PERTUSSIS* VACCINATED RAT

Conc. of Histamine (mg./l.)	% Paralysis	
	Normal Animal	Vaccinated Animal
1	0	0
10	0–8	4–13
100	8–21	9–23
300	8–44	—
1,000	20–40	—

DISCUSSION

The observed effects of histamine on the heart rate and ECG of the normal rat may result from coronary insufficiency and a change in ionic equilibrium. The action on plain muscle is shown by a bronchoconstriction of relatively minor importance, the central action is characterized by a desynchronization and a flattening of the EEG tracing, and large doses decrease the height of the contractions of the masseter and gastrocnemius muscles.

Under these conditions, the multiplicity of the factors which enter into histamine intoxication was confirmed. Death in the normal animal on rapid intravenous injection of 500 mg./kg. histamine or more took place almost instantaneously and would appear to be due mainly to cardiovascular collapse. Doses of histamine above 500 mg./kg. (i.v.) were invariably lethal as were those of Mayer and Brousseau (1946), Feldberg and Schilf (1930) and Bovet and Bovet-Nitti (1948). It is of particular interest that, whereas the normal animal receiving an intravenous administration of a lethal dose of histamine dies immediately, the vaccinated animal when treated with its lethal dose (20 mg./kg. or more) takes longer to die. Death of the vaccinated rat was not directly due to asphyxia, for the animal was not protected from the lethal effects of histamine by artificial respiration.

The immediate and considerably increased response of the vaccinated rat to the neuromuscular action of histamine was characteristic. Whereas the dose required in the normal animal to produce some effect on the masseter muscle was from 200 to 500 mg./kg. (i.v.), the vaccinated rat was sensitive to a dose of 5 mg./kg. (i.v.). The ratio here (from 40 to 100:1) agreed with other estimates for enhanced sensitivity quoted in the literature. Thus Parfentjev and Goodline (1948) obtained 25-fold and Gauthier, Loew, and Jenkins (1955) a 33-fold increase in the toxicity after pertussis vaccination.

Reports upon the effects of histamine on skeletal muscle and neuromuscular transmission in the rat are very scanty. Sömjén and Uyldert (1955) recently showed that the histamine liberator 48/80 provoked a distinct diminution in the contraction of the gastrocnemius muscle of the rat, but the action of histamine on this preparation was not investigated. These authors were of the opinion that the effects observed were due to the action of the compound itself and not to liberated histamine. Feldberg (1956) has suggested that histamine in the rat may give rise to a pronounced muscular weakness and paralysis, either by direct muscular action or by producing neuromuscular block. It is clear from the experiments here reported that histamine does, in fact, exert an effect on the muscular contraction of the rat, but the mechanism of the block produced is not clear.

In discussing possible explanations for the markedly increased sensitivity of the vaccinated rat to histamine it is of interest to record the recent work of Macmillan and Vane (1956), who examined the effect of histamine on the plasma potassium level of the cat. These authors suggest that the intravenous injection of histamine causes a leak of potassium into the plasma which gives rise to the pharmacological effects, and that potassium may play a significant rôle in histamine intoxication. The possibility cannot therefore be excluded that, in the vaccinated rat, histamine may cause changes in the ionic equilibrium. In this connexion it may be remembered that the vaccinated, like the adrenalectomized animal, may be protected from fatal shock by pretreatment with cortisone. The importance and the influence of the suprarenal hormones on the mineral balance are well known.

An investigation of the effects of histamine on the potassium level in the normal and vaccinated animal is in progress.

We should like to express our thanks to Dr. J. Ungar of Glaxo Laboratories Ltd., Greenford, Middlesex, for a generous supply of *H. pertussis* vaccine.

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EFFECTS OF RAUNESCINE AND ISORAUNESCINE ON BEHAVIOUR AND ON THE 5-HYDROXYTRYPTAMINE AND NORADRENALINE CONTENTS OF BRAIN

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Some behavioural effects of raunescine and isoraunescine on pigeons have been studied; no qualitative difference was detected between their effects and those of reserpine. Isoraunescine is between five and ten times less potent than raunescine, which, in turn, is much less potent than reserpine in producing these effects.

Both raunescine (5 mg./kg.) and isoraunescine (50 mg./kg.) were found to cause a reduction in the concentration of noradrenaline in the brains of rats. Raunescine (5 mg./kg.) also caused a reduction in the concentration of 5-hydroxytryptamine in brain; isoraunescine did not do so in the same dose; higher dose levels were not studied.

In a study of the effects of a number of Rauwolfia alkaloids on the adrenaline and noradrenaline contents of the hearts of rats, it was found that both raunescine and isoraunescine caused a fall in the noradrenaline content (Krayer and Paasonen, unpublished observations).

It has been reported that following administration of reserpine the disappearance and restoration of noradernaline in rabbit brain run parallel to that of 5-hydroxytryptamine. Since it has been suggested that the behavioural effects of Rauwolfia alkaloids may be due to their ability to release from brain 5-hydroxytryptamine (Hess, Shore, and Brodie, 1956) and/or noradrenaline (Holzbauer and Vogt, 1956), the effects of raunescine and isoraunescine both on the 5-hydroxytryptamine and catechol amine content of brain and on behaviour have been studied. For technical reasons, the chemical studies were conducted on rats and the behavioural studies on pigeons.

MATERIALS AND METHODS

Behaviour.—For the experiments on behaviour, pigeons were maintained at a relatively constant level of hunger by keeping them at 80% of the body weight which they attained when fed *ad libitum*. Each day they were exposed to a fixed succession of stimuli in an experimental space. When the behavioural performance had stabilized, observations on the effects of drugs on the performance were started. Details of the experimental procedure have been published (Dews, 1955). Briefly, the pigeons were trained to peck a translucent plastic disk (the key). Each peck broke electrical contacts causing a relay to release. A response is defined as a peck to the key with this consequence. Sometimes, according to a definite schedule, a response led to the presentation of grain to the pigeon for 4 sec. One schedule (designated schedule 14) used was the "multiple schedule" that has been used in previous studies (Dews, 1956). When a red light was present behind the key, grain was presented at the 50th response made (FR); while when a blue light was present, grain was presented at the first response made after an interval of 15 min. had elapsed (FI). The sequence of lights was: R(ed)RRB(lue)RBBRRRRRRRRRRBBR, the changes occurring at food presentation. Five birds were studied on schedule 14. The second schedule (designated schedule 15) was identical with schedule 14 except that the same light was present behind the key, irrespective of whether FR or FI contingencies were in operation. Four birds were studied on schedule 15. Finally, 2 birds were given food at every 50th peck made during a period of 15 min. (FR 50; Dews, 1955). Results were obtained in two forms: as an automatically recorded graph of cumulative number of pecks (ordinate) against time (abscissa) (Figs. 1 and 2) and also as total numbers of pecks made as read from a digital counter.

Brain Chemistry.—For the experiments on brain chemistry, white male rats weighing 160 to 270 g. were used. Whole brains (with the exception of cerebella which were excluded) were removed following decapitation, and were homogenized in saline. The extraction of 5-hydroxytryptamine was done accord-

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ing to the suggestion of Amin, Crawford, and Gaddum (1954) in 95% acetone. Fats were removed by petroleum ether, and the 5-hydroxytryptamine assayed biologically on the heart of *Venus mercenaria*. The conditions and procedure were as described by Paasonen and Vogt (1956) except that real, instead of artificial, sea water was used. For catechol amines, the excised brain was first homogenized with 10% trichloracetic acid and then subjected to the procedure described by v. Euler (1956) with minor changes (Krayer and Paasonen, unpublished observation). The final estimation was done biologically on the blood pressure of the cat and the isolated rectal caecum of the hen (v. Euler, 1948). The cats were given the combination of drugs used by Kärki (1956) before start of the assay.

Reserpine, raunescine, and isoraunescine were dissolved in glacial acetic acid and the solution diluted so that the concentration of acetic acid injected was not in excess of 1%. The drugs were administered intramuscularly to pigeons and intraperitoneally to rats. Control experiments were made using the vehicle alone. In the pigeon experiments, the doses specified are the total doses administered.

RESULTS

Qualitative Considerations

Behavioural Effects.—On schedule 14, the pigeons behaved quite differently according to whether the red or the blue light was on. While the red light was on, the pigeon pecked continuously at a high rate. When the blue light came on, the normal pigeon waited some time before making any response; once it had started to peck there was a period of acceleration followed by sustained pecking at a more or less constant rate until food was presented. Bearing in mind that the slope of the cumulative curve gives the rate of pecking, these various features of the normal performance can be seen in Fig. 1, A. These findings are in full agreement with previously published descriptions (Dews, 1956; Ferster and Skinner, 1957).

The FR performance (the high fixed rate of pecking in the presence of the red light) was much less sensitive to modification by the drugs than was the generally lower and progressively changing rate of pecking characteristic of the FI performance (Fig. 1, B, C, D, E). This same differential sensitivity has been previously described with other drugs (Dews, 1955, 1956). In fact, in none of the experiments illustrated in Fig. 1 did the FR performance differ appreciably from the control: hence the changes in the FI performances cannot have been due to effects of the drugs on the "physical" ability of the birds to peck such as might result, for example, from ataxia.

The effects of raunescine and isoraunescine on FI performance were similar. The normal initial pause succeeded by steady pecking up to the end of the interval was converted to irregular bursts of pecking at a high rate separated by more or less

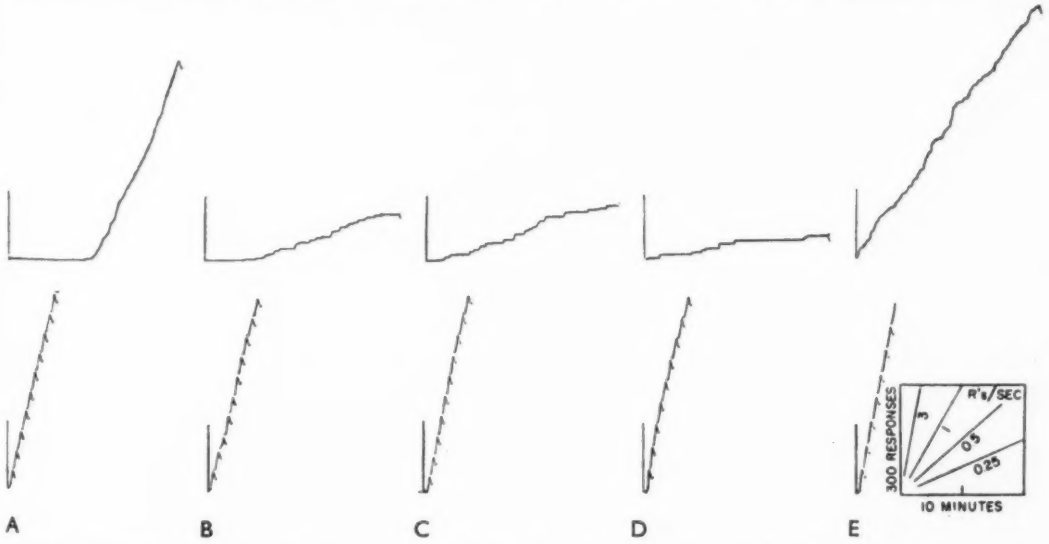

FIG. 1.—The effects of drugs on performance on schedule 14. The upper series of curves shows the cumulative response/time graph (recorded automatically) for the second presentation of the blue light (FI contingency) on a number of days. The lower series shows the sequence of 10 consecutive presentations of the red light (FR contingency) on the same day. The small diagonal hatch marks show the times of food presentation. All records were obtained from the same bird. Times are given to start of the FI contingency. A, control in which no drug was given. B, 30 min. after 1 mg. of raunescine. C, 1 hr. 45 min. after 1 mg. of isoraunescine. D, 3 hr. after 0.05 mg. of reserpine. E, 15 min. after 5·2 mg. of sodium pentobarbitone.

prolonged periods with no pecks (Fig. 1, B, C). The total number of pecks made was greatly reduced. Reserpine had similar effects (Fig. 1, D), but the effects of pentobarbitone were quite different (Fig. 1, E). As previously described, pentobarbitone in appropriate doses caused sustained though irregular pecking throughout the interval (Dews, 1955; Herrnstein and Morse, 1957).

The normal birds on schedule 15 always started pecking at a high rate as soon as the key light appeared. If the food was presented at the fiftieth peck, the 50 pecks were made before a pause occurred. On the occasions when food was presented at a peck only when 15 min. had elapsed, the initial high rate was followed by an abrupt break, and then a succession of pecking rates quite similar to the FI performance of schedule 14 (Fig. 2, F). Purely for the purposes of description, and without prejudice, the following anthropomorphic account can be given. The colour of the key light on schedule 15 gave no information as to whether reward was to occur after 49 pecks or only after 15 min. The pigeon therefore pecked at a high rate until either reward occurred on the fiftieth peck, or until more than 50 pecks had been made without reward, thus indicating that the reward would occur only after the end of the 15 min. period, at which time the FI performance started.

The effects of raunescine and isoraunescine were again to lead to a greatly increased tendency to pause (Fig. 2, G, H), and are indistinguishable from the effects of reserpine (Fig. 2, J). The doses and time intervals for the isoraunescine and reserpine experiments illustrated were such that the "FR" performance was also disturbed (Fig. 2, H, J); the tendency again was for pauses to develop (Dews, 1956).

Quantitative Considerations

The mean number of responses in the FI components of control experiments on schedule 14 was 2,742 and on schedule 15 was 3,674; the coefficients of variation for individual birds averaged 0.22 and 0.18 respectively.

Although effects of both raunescine and isoraunescine were apparent 15 min. after injection (Fig. 1, B, and Table I, pigeons 66 and 165), the maximum effect was not obtained until at least 1.5 hr. had elapsed. A dose of 1 mg. raunescine abolished all responding in experiments on schedule 15 starting 4.25 and 6.75 hr. after injection, and a dose of 0.3 mg. raunescine abolished all responding in an experiment on schedule 14.

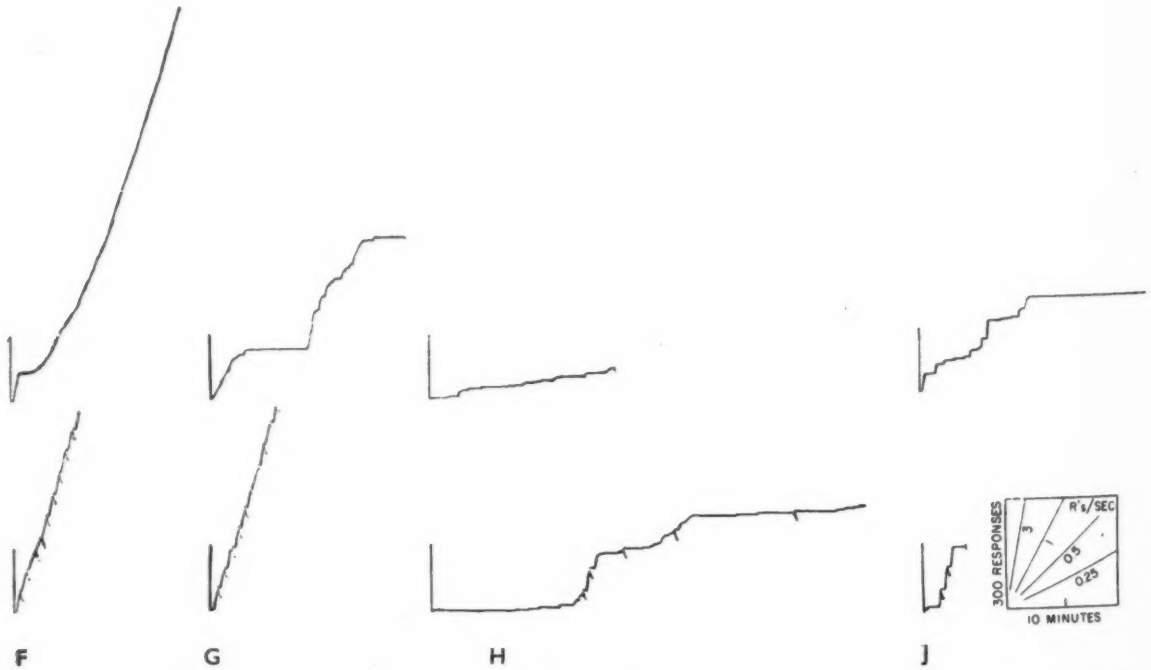

FIG. 2.—The effects of drugs on performance on schedule 15. The general arrangement of this figure is identical with that of Fig. 1, performance under FI contingencies being shown above, and under FR below, although the key light stimuli were constant throughout the experimental session. F, control in which no drug was given. G, 5 hr. 45 min. after 0·1 mg. of raunescine. H, 5 hr. 45 min. after 1·0 mg. of isoraunescine. J, 5 hr. 45 min. after 0·05 mg. of reserpine.

starting 1.5 hr. after injection (Table I). In none of nine experiments, started at various times after injection, did 1 mg. isoraunescine abolish responding. This suggests that raunescine has at least three times the potency of isoraunescine in this regard. On the other hand, in all of four pairs of experiments in which 0.1 mg. of raunescine and 1 mg. of isoraunescine were compared in the same bird at similar times after injection, 1 mg. of isoraunescine showed the greater activity (Table I).

TABLE I

EFFECTS OF RAUNESCINE AND ISORAUNESCINE ON BEHAVIOUR OF PIGEONS

The weight recorded in column 2 is the "running weight" and is about 80% of the weight attained on *ad lib.* feeding. Column 5 records the time between the injection of the drug and the start of the experiment. "Output ratio" is defined as the number of pecks made by the bird while the blue light was on after injection of the drug, divided by the average number made during the blue light on 10 control days. For the description of schedules see text.

Bird (1)	Weight (g.) (2)	Schedule (3)	Dose (mg.) and Drug (4)	Time (hr.) (5)	Output Ratio (6)
66	420	14	1·0 raunescine	0·25	0·10
66	420	14	1·0 isoraunescine	1·5	0·18
1	370	14	0·1 raunescine	3·0	0·74
1	370	14	1·0 isoraunescine	3·0	0·57
165	410	14	1·0 ,,	0·25	0·25
156	440	14	0·3 raunescine	1·5	0
157	500	14	1·0 isoraunescine	3·0	0·10
55	430	15	1·0 raunescine	4·25	0
55	430	15	1·0 isoraunescine	4·25	0·01
62	420	15	0·1 raunescine	5·5	0·84
62	420	15	1·0 isoraunescine	5·5	0·11
166	460	15	1·0 raunescine	6·75	0
166	460	15	1·0 isoraunescine	6·75	0·74
175	400	15	0·1 raunescine	8·0	1·28
175	400	15	1·0 isoraunescine	8·0	0 81
169	420	FR	0·1 raunescine	2·75	0·74
169	420	FR	1·0 isoraunescine	2·75	0·57
172	460	FR	1·0 raunescine	2·75	0·05

Thus, raunescine would appear to have less than 10 times the potency of isoraunescine. Since 0.1 mg. reserpine abolishes all responding for prolonged periods (Dews, 1956), reserpine is, in turn, much more potent than raunescine (probably about 10 times more potent). The results of the three experiments on FR 50 were in agreement with the suggested relative potencies of raunescine and isoraunescine.

Effects on Brain Chemistry.—The concentration of 5-hydroxytryptamine in the brains of rats fell to about half its control value 6 hr. after administration of 5 mg./kg. raunescine. Isoraunescine had no effect on 5-hydroxytryptamine concentration at this dose (Table II). The concentration of noradrenaline was reduced to about one-eighth of its control value by 5 mg./kg. raunescine, and again the same dose of isoraunescine had no effect. However, 50 mg./kg. isoraunescine caused a great reduction in noradrenaline concentration, although probably not as great a fall as that produced by 5 mg./kg. of raunescine. This suggests that

TABLE II

EFFECTS OF RAUNESCINE AND ISORAUNESCINE ON 5-HYDROXYTRYPTAMINE, NORADRENALINE, AND ADRENALINE CONTENTS OF BRAINS OF RATS

Amine content is given in ng./g. wet weight of brain. The time recorded is the interval between injection of the drug and removal of the brain.

Drug	Dose (mg./kg.)	Time (hr.)	Individual Values	Mean and Standard Deviation
			5-Hydroxytryptamine	
Controls ..			345, 348, 368, 385, 386	366 ± 19·6
Raunescine	5	6	139, 160, 168, 230	174
,,	5	16	178, 205, 226, 297	227
Isoraunescine	5	6	350, 352, 360, 412	369
,,	5	16	330, 370, 373, 380	363
			Noradrenaline	
Controls ..			172, 175, 187, 200, 212, 220, 262	204 ± 31·1
Raunescine	5	6	14, 23, 28, 35	25
,,	5	16	18, 46, 53, 70	47
Isoraunescine	5	6	175, 212, 218, 240	211
,,	5	16	220, 230, 232, 265	237
,,	50	6	25, 47, 69, 93	59
			Adrenaline	
Controls ..			10, 17, 20, 26, 31	21 ± 8·1
Raunescine	5	6	11, 14, 13, 18	14
,,	5	16	17, 18, 26, 26	22
Isoraunescine	5	6	7, 15, 16, 18	14
,,	5	16	9, 16, 23, 29	19

raunescine is more than 10 times as potent as isoraunescine in causing this effect. On the other hand, it has been previously shown that 2.5 mg./kg. of reserpine depleted about 80% of brain 5-hydroxytryptamine (Paasonen, unpublished observation), so reserpine is much more potent than raunescine in causing this effect.

The effect of raunescine on the adrenaline content of brain was much less than its effect on noradrenaline.

DISCUSSION

In sufficient dosage, both raunescine and isoraunescine caused behavioural effects which had many similarities to those of reserpine. With the procedure used, the effects of all three drugs were qualitatively indistinguishable. Raunescine and isoraunescine, also like reserpine, caused a reduction in the concentration of both 5-hydroxytryptamine and noradrenaline in the brains of rats. Much larger doses of raunescine and isoraunescine were required to cause the biochemical changes in rats than were required to cause the behavioural effects in pigeons. However, much larger doses of reserpine are required to cause *behavioural* effects in rats than are required in pigeons. While 1 mg./kg. of reserpine was required to abolish lever-pressing behaviour in rats almost completely (Sidman, 1956), about one-fifth of this dose completely abolished all

pecking behaviour in pigeons for 18 hr. (Dews, 1956). The order of potency of the three compounds is reserpine>raunescine>isoraunescine, both behaviourally and biochemically.

The dose of 5 mg./kg. of raunescine caused greater reduction in the brain noradrenaline concentration (to 10% of control) than it did in brain 5-hydroxytryptamine concentration (to 50% of control). Dissociation of noradrenaline and 5-hydroxytryptamine release in this fashion may make it possible to identify which of these two (if either) is related to the behavioural effects of the drugs. The results of these studies are compatible with the postulated indirect effect of the appropriate Rauwolfia alkaloids on the brain, namely that they exert their effect by release of noradrenaline or 5-hydroxytryptamine, and lend some support to this viewpoint.

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FORMATION OF HISTAMINE IN THE KIDNEY OF THE DOG

BY

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In dogs [^{14}C]L-histidine was given in a steady infusion into one renal artery while urine was collected from both kidneys separately. The urine from the infused kidney contained considerably more [^{14}C]histamine than did the urine from the other kidney. The difference between the kidneys in the amounts of [^{14}C]histamine appearing in the urine was considered to be due to formation of histamine from histidine in the injected kidney. It is concluded that histamine formed in the kidney may contribute to the urinary histamine.

The source of the urinary histamine has not been definitely established. It has been proposed that the urinary histamine has been carried to the kidney in the plasma (Adam, Card, Riddell, Roberts, and Strong, 1954). On the other hand, evidence has been presented that L-histidine may be decarboxylated in the kidney of the rat and that the resulting histamine is excreted without having entered the circulation (Schayer, Wu, and Smiley, 1954).

The present study was undertaken to find out if histamine could be formed from histidine in the kidney of the living dog.

METHODS

The dogs were anaesthetized with pentobarbitone sodium (30 mg./kg.) given intravenously. Under fluoroscopy, a polythene catheter was guided into one of the renal arteries from a femoral artery (Lindell and Olin, 1957). 5 mg. of [^{14}C]L-histidine (activity 10 microcuries/mg.) was dissolved in a solution containing 0.9% NaCl and a small amount of HCl so that the *p*H was approximately 5. This solution was injected with a motor-driven syringe into the catheter in the renal artery at a rate of 25 to 30 μg. histidine/min. for 160 to 200 min. The [^{14}C]L-histidine solution which remained in the syringe at the end of the infusion was assayed for [^{14}C]histamine in the same way as the urine samples described below. It did not contain any measurable amount of [^{14}C]histamine. During the infusion of histidine, urine was collected from polythene tubes inserted into the ureters. The urine was collected in glass tubes containing 0.5 ml. 6 N-HCl. The volumes were measured and aliquots taken for the assays.

Assay of [^{14}C]Histamine, Methylhistamine {(4-(2-aminoethyl)-1-methylimidazole}, methylimidazoleacetic acid (1-methylimidazol-4-ylacetic acid) and imidazoleacetic acid.—The histamine samples were extracted as described by Schayer, Davis and Smiley (1955). The histamine dipicrates prepared were converted to pipsylhistamine before counting. All assays were performed as described by Lindell and Schayer (1958a).

Histamine showed constant radioactivity after the second recrystallization as the pipsyl derivative. The activity of the methylimidazoleacetic acid samples was not constant until after the sixth recrystallization as the picrates. The imidazoleacetic acid samples reached constant radioactivity after the ninth recrystallization as the pipsyl derivative.

RESULTS

The results of two experiments have been summarized in Table I. It may be seen that the urine from the injected kidney contained several times more [^{14}C]histamine than did the urine from the

TABLE I

ANALYSIS OF [^{14}C]HISTAMINE AND [^{14}C]METHYLHISTAMINE IN URINE FROM TWO EXPERIMENTS IN DOGS

In both experiments, 5,000 μg. of [^{14}C]L-histidine was given in a steady infusion into one renal artery while urine was collected from each kidney separately. Time of infusion in dog 1, 160 min., and in dog 2, 200 min. One μg. of the histamine formed from this histidine counted as the pipsyl derivative under standard conditions (40 mg. histamine base as carrier, in plates of 4 cm.^{2} at infinite thickness in a flow counter), gives 580 c./min. above background.

	[^{14}C]Histamine in Urine Collected during Infusion (c./min.)	[^{14}C]Methylhistamine in Urine Collected during Infusion (c./min.)	Weight of Kidney (g.)	Volume of Urine Collected from the Ureter (ml.)
1. Male dog (9 kg.)				
Injected kidney	240	0	19	17
Control ,,	0	0	20	18
Difference ..	240	0		
2. Male dog (16 kg.)				
Injected kidney	380	0	40	18
Control ,,	90	0	38	16
Difference ..	290	0		

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other kidney. The difference may be interpreted as due to formation of histamine from histidine in the injected kidneys. In the first experiment, the value of 240 c./min. (difference between injected kidney and the kidney of the opposite side) is equal to about 0.4 μg. [^{14}C]histamine. In the second experiment, the difference is 290 c./min. or roughly 0.5 μg. [^{14}C]histamine. No methylhistamine could be detected in any of the urine samples.

[^{14}C]Methylimidazoleacetic acid and [^{14}C]imidazoleacetic acid were also determined in the urine samples. Their determination by the isotope dilution technique is, however, much more difficult than the determination of histamine and methylhistamine. This is so because of the presence of a huge excess of histidine and histidine metabolites, some of which are imidazolecarboxylic acids and have properties similar to methylimidazoleacetic acid and imidazoleacetic acid. For this reason a more rigorous establishment of purity is required before the values can be considered valid. The activity of the methylimidazoleacetic acid samples from these dog urine samples was about the same as the activity of the histamine samples. The imidazoleacetic acid samples, however, were about a hundred times more active than the histamine samples.

Discussion

Considerably more [^{14}C]histamine appeared in the urine from the infused kidney than in the urine from the other kidney. Since the histidine solution did not contain any measurable amounts of [^{14}C]histamine it seems likely that the excess [^{14}C]histamine in the urine from the infused kidney was formed in that kidney. In this connexion, it should be mentioned that the catheter used for the injections into the renal artery did not influence the clearance of phenol red neither on the catheterized side nor on opposite side (Lindell and Olin, 1957). Also it did not change the renal extraction and excretion of *p*-aminohippuric acid (Lindell, unpublished observation).

When [^{14}C]histamine was injected into dogs, [^{14}C]methylhistamine appeared in the urine in amounts which were of the same order of magnitude as the amounts of [^{14}C]histamine in the urine (Lindell and Schayer, 1958a and b). The absence of [^{14}C]methylhistamine in the urine samples from the present experiments might therefore indicate that very little [^{14}C]histamine reached the kidneys with the blood stream.

The amounts of [^{14}C]methylimidazoleacetic acid and imidazoleacetic acid appearing in the urine of dogs after injection of [^{14}C]histamine are usually of the same order of magnitude. In the present experiments where [^{14}C]L-histidine was injected the amounts of [^{14}C]imidazoleacetic acid in the urine were about a hundred times greater than the amounts of [^{14}C]methylimidazoleacetic acid. This suggests that in the dog imidazoleacetic acid can be formed from histidine by a pathway not involving histamine. Such a pathway has been reported to occur in rats (Wolf, Wu, and Heck, 1956).

In the present experiments, evidence was obtained that histamine was formed from histidine in the kidney of the dog. Since histidine occurs in the plasma of the dog (Wright, Russo, Skeggs, Patch and Beyer, 1947) it seems possible that some of the histamine that appears in the urine has been formed in the kidney.

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THE ACTION OF DOPAMINE ON THE ARTERIAL BLOOD PRESSURE OF THE GUINEA-PIG

BY

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The depressor action of dopamine (β-3 : 4-dihydroxyphenylethylamine) upon the arterial blood pressure of the guinea-pig has been studied. This effect begins without a latent period. It is often enhanced after the intravenous injection of iproniazid (Marsilid). The depressor response is sufficiently sensitive to serve as a method of bioassay of dopamine in microgram quantities. Observations on the depressor action of L-dopa have also been made. This effect is also enhanced by iproniazid; it begins after a latent period. Epinine (β-3 : 4-dihydroxyphenylethylmethylamine) caused a pressor response, followed by a fall of arterial blood pressure. No evidence was obtained in support of the suggestion that the two amines, which are oxidized at similar rates by amine oxidase, cause a fall of blood pressure after their conversion to an aldehyde by the action of amine oxidase.

In addition to noradrenaline and adrenaline, there occurs in mammals a third catechol amine; this is dopamine (hydroxytyramine, β-3 : 4-dihydroxyphenylethylamine) (Goodall, 1951). It is now known that dopamine is the immediate precursor in the formation of noradrenaline in chromaffin tissue and adrenergic nerves.

The biological activity of dopamine has been known for a long time (Barger and Dale, 1910; Gurd, 1937; Holtz, Heise and Lüdtke, 1939). In most preparations, the amine has sympathomimetic activity, but it is less potent than adrenaline or noradrenaline.

A response to dopamine which differs qualitatively from that to the other two naturally occurring catechol amines was described by Holtz and Credner (1942). These authors made a comparative study of the action of dopamine on the arterial blood pressure in different species, and they found that, in the guinea-pig and, to a lesser extent, in the rabbit, dopamine caused a fall in the arterial blood pressure. This finding has been confirmed by Schümann (1956), who used the lowering of the blood pressure in the guinea-pig for the determination of dopamine in extracts of splenic nerves and sympathetic ganglia (see also Dengler, 1957). The fall of the blood pressure after administration of dopamine was ascribed by Holtz and Credner (1942) to the action of the aldehyde produced when dopamine is oxidized by the enzyme amine oxidase. It is known that dopamine is very rapidly oxidized by this enzyme (Blaschko, Richter, and Schlossmann, 1937).

The fact that dopamine has an action different from that of the other two catechol amines is of interest. It is at present not known if dopamine has any regulatory functions in addition to its metabolic rôle as the immediate precursor of noradrenaline.

The experiments to be described were undertaken in order to find out, firstly, if the response of the arterial blood pressure of the guinea-pig to dopamine could serve as the basis for a method of assay and, secondly, whether support for the rôle of amine oxidase in the depressor response to dopamine in the guinea-pig could be obtained.

METHODS

Male guinea-pigs of 400 to 650 g. were anaesthetized with ethyl urethane (2 g./kg. body weight given subcutaneously). A polythene tube was introduced into the right jugular vein and heparin, 10 mg./kg., was administered. The left carotid artery was cannulated and the blood pressure was recorded, using the mercury manometer described by Condon (1951). All substances were administered intravenously and the volume injected was kept constant in each experiment; usually it was 0.3 ml. All doses are given in terms of the base.

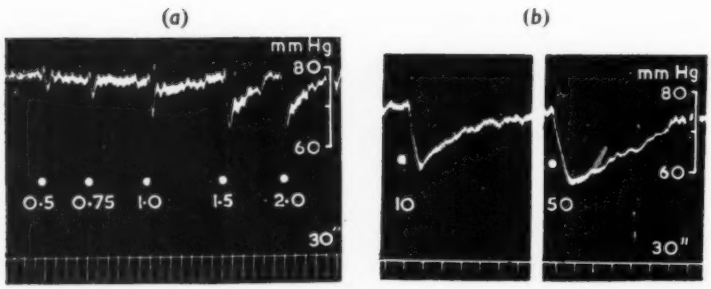

FIG. 1.—Effect of dopamine on the arterial blood pressure of the guinea-pig. (*a*), Guinea-pig, 450 g. urethane anaesthesia. Response of the arterial blood pressure to increasing doses (0·5 μg. to 2·0 μg.) of dopamine, injected intravenously. (*b*), Guinea-pig, 600 g. urethane anaesthesia. Effect of large doses (10 μg. and 50 μg.) of dopamine.

RESULTS

Relation Between Dose and Response.—The response of the arterial blood pressure of the guinea-pig to different doses of dopamine is shown in Fig. 1. In Fig. 1*a*, doses from 0.5 to 2.0 μg. were given ; the depressor response increased with increasing dose. Fig. 1*b* is from another animal in which doses of 10 μg. and 50 μg. were given. In all these experiments the fall of blood pressure was immediate, without any sign of a latent period.

Effect of L-*Dopa and Epinine.*—Experiments were also carried out in which L-dopa was injected. In agreement with observations by Holtz and Credner (1942) dopa caused a fall in the arterial blood pressure, but this began after a brief latent period and was more protracted than with dopamine. The typical response to a small dose of dopa (200 μg.) is shown in Fig. 2.

Fig. 3 shows the typical response to an injection of 10 μg. of epinine (β-3 : 4-dihydroxyphenylethylmethylamine). The immediate response was a rise of the arterial blood pressure followed by a fall, similar in duration to that caused by dopamine.

Effect of Iproniazid.—In a number of experiments iproniazid was given in order to inhibit the action of amine oxidase on dopamine. Dopamine

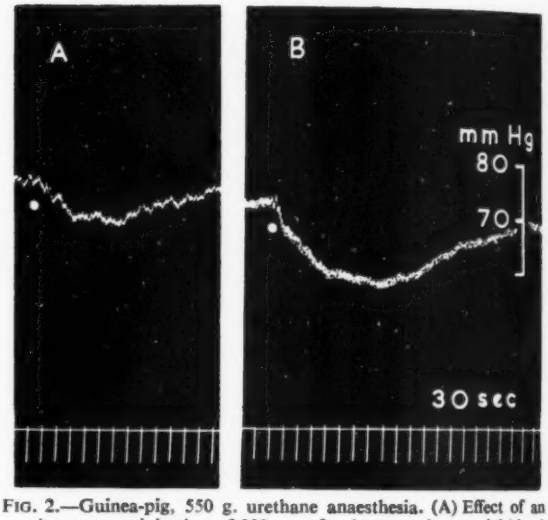

FIG. 2.—Guinea-pig, 550 g. urethane anaesthesia. (A) Effect of an intravenous injection of 200 μg. of L-dopa on the arterial blood pressure; (B) effect of the same dose of dopa, 90 min. after iproniazid, 40 mg./kg. intravenously.

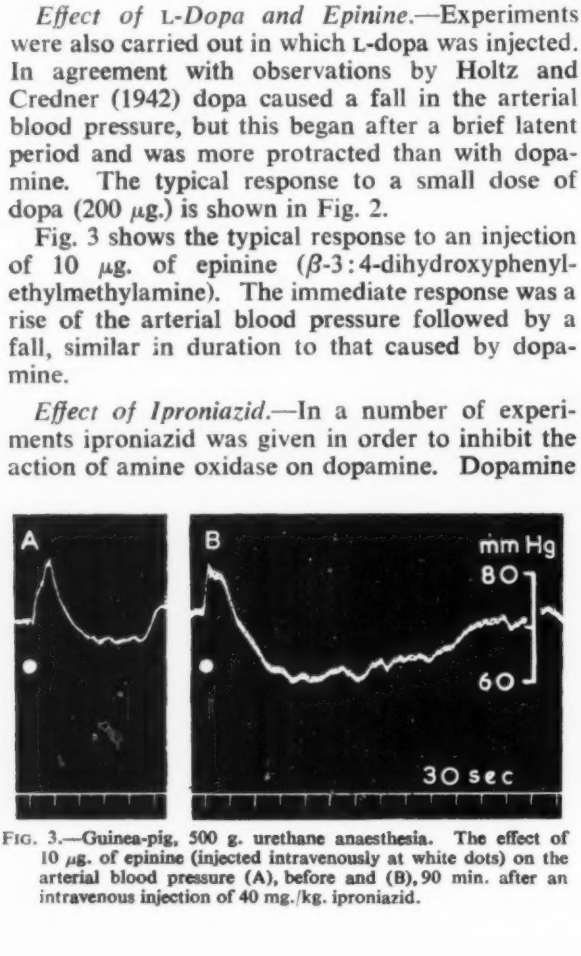

FIG. 3.—Guinea-pig, 500 g. urethane anaesthesia. The effect of 10 μg. of epinine (injected intravenously at white dots) on the arterial blood pressure (A), before and (B), 90 min. after an intravenous injection of 40 mg./kg. iproniazid.

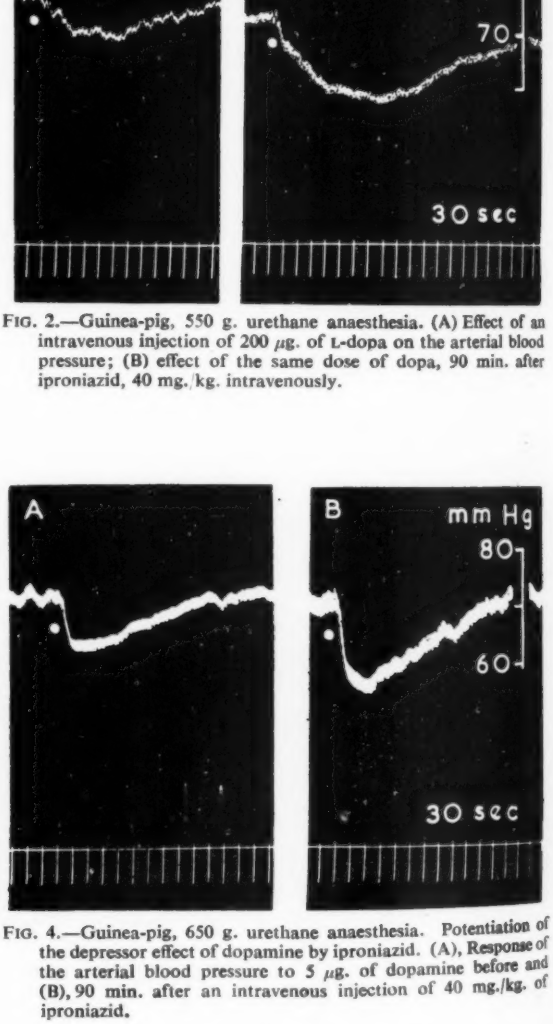

FIG. 4.—Guinea-pig, 650 g. urethane anaesthesia. Potentiation of the depressor effect of dopamine by iproniazid. (A), Response of the arterial blood pressure to 5 μg. of dopamine before and (B), 90 min. after an intravenous injection of 40 mg./kg. of iproniazid.

was injected before, and 90 min. after, an intravenous injection of 40 mg./kg. of iproniazid.

In none of these experiments was there a diminished depressor response to dopamine after iproniazid ; in most the response to dopamine after iproniazid was enhanced and sometimes prolonged. Fig. 4 shows the response to 5 μg. of dopamine before and after iproniazid.

Experiments on the effect of iproniazid on the depressor action of dopa were also carried out. Here, too, an enhancement of the depressor response usually occurred. This can be seen in Fig. 2, where 0.3 mg. of dopa was injected.

Oxidation Products of Dopamine and Epinine.—In order to obtain information on the action of the products of oxidation of the two amines by amine oxidase, a manometric experiment was carried out in which an enzyme preparation was incubated with either epinine or dopamine ; the incubation was terminated just after about 0.5 molecule of oxygen/molecule of substrate had been taken up. For this experiment an acetone-dried preparation of guinea-pig liver was used. Each manometer flask contained 50 mg. of powder, thoroughly washed with 0.067 M-sodium phosphate buffer of *p*H 7.4. At the zero moment, 0.2 ml. of an 0.05 M solution of amine hydrochloride was tipped in from the side arm of a conical manometer flask. The total volume of fluid in each flask was 2.0 ml.; the gas phase was O_2 and the temperature was 37.5°.

The initial rates of oxygen uptake with dopamine and epinine were similar. Two vessels, one with dopamine, the other with epinine, were removed after 27 min., when the oxygen uptake was 127 and 138 μl. respectively. The samples were quickly centrifuged at 0°, and fractions, suitably diluted, were injected intravenously. Another pair of samples was incubated for 39 min., when the uptake of oxygen was 141 and 155 μl. respectively.

The samples which had been incubated with dopamine caused a very slight fall in blood pressure ; the samples incubated with epinine caused a rise, followed by a fall, similar to that produced by the injection of an aqueous solution of epinine. However, the size of the responses to the incubated samples was much less than that to the injection of the corresponding amount of amine not incubated with a preparation containing amine oxidase. In other words, there was no indication that the fall of arterial blood pressure was greater after incubation.

Action of Dopamine on the Heart Rate.—The heart rate before and after an injection of dopamine was recorded by means of an electrocardiograph (Lead II). There was no change in the rate after intravenous injection of 2 μg. of dopamine ; after 100 μg. the rate fell from 280 beats/min. to 260.

Discussion

The experiments described in this paper confirm earlier observations, in which it was shown that dopamine lowers the arterial blood pressure in the guinea-pig. The depressor effect was constant and could usually be elicited by relatively small amounts of the amine. This shows that this effect can serve as the basis of a quantitative determination of dopamine.

The cause of the depressor effect is not known. Holtz and Credner (1942) suggested that it was due not to the action of dopamine itself, but to that of the aldehyde formed from it by enzyme action. It is difficult to reconcile this interpretation with some of the observations reported. Firstly, the response was prompt, without any visible latent period. Secondly, the response to epinine differed from that to dopamine, although both amines are oxidized by guinea-pig amine oxidase at a similar rate (Blaschko *et al.*, 1937 ; Beyer, 1943 ; Randall, 1946). This was confirmed in the present experiments. The two amines should give rise to the same aldehyde as the primary product of oxidation. Thirdly, iproniazid, an inhibitor of amine oxidase, did not abolish or diminish the depressor reaction ; on the contrary, in most experiments, the fall of blood pressure was enhanced and prolonged after iproniazid. It has recently been questioned whether iproniazid is as effective an inhibitor of amine oxidase *in vivo* as it is *in vitro* (Udenfriend, Weissbach, and Bogdanski, 1957). That the drug does inhibit the breakdown of dopamine *in vivo* is supported by observations of Balzer and Holtz (1956), who have shown that in guinea-pigs treated with iproniazid the amounts of dopamine excreted in the urine after administration of dopa were almost doubled. In the present experiments, iproniazid enhanced the depressor response to dopa whenever the response to dopamine was potentiated.

The depressor action of dopamine was not due to an effect on the heart rate because doses which caused a marked fall of blood pressure were without significant action upon the heart rate.

I wish to express my thanks to Professor J. H. Burn, F.R.S., for the opportunity of working in his department and to Dr. H. Blaschko for his help in the manometric experiment and for his constant advice and encouragement. The work was done during the tenure of a British Council Scholarship.

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THE INFLUENCE OF CORTISONE AND HYDROCORTISONE ACETATES ON THE COURSE OF *MYCOBACTERIUM LEPRAEMURIUM* INFECTION IN RATS

BY

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Prolonged administration of cortisone acetate or hydrocortisone acetate produces a decrease in the rate of growth of treated rats. In this respect the effect of the latter steroid is the more pronounced. Lepromata in the steroid treated animals are significantly smaller than those in the control rats. This difference is more pronounced with the cortisone acetate treatment. However, whilst there is no appreciable difference in the distribution of organisms in the lepromata, the spread of infection is not increased by the steroid treatment, as the number of animals showing acid fast organisms in the spleen is no greater in the treated groups than in the controls.

It has been repeatedly demonstrated that corticotrophin (ACTH), cortisone acetate and some other adrenal steroids, when injected in large doses, will interfere with the host defence mechanism against bacterial infections (Foley, Morgan, and Greco, 1957a). It has also been suggested that the steroids interfere both with antibody formation and with the development of tissue immunity (Foley *et al.*, 1957a and b). In addition, other investigators have shown that cortisone will lower the resistance of laboratory animals to a wide range of bacterial, viral, protozoal and fungal infections (Kass and Finland, 1953).

The majority of experiments in this field reported in the literature have been of short duration. It was therefore thought of interest to investigate the effect of prolonged administration of cortisone and hydrocortisone acetates on the course of *Mycobacterium lepraemurium* infection in rats, which affords an excellent opportunity of studying the effect of administering the steroids in an infection of long duration.

MATERIALS AND METHODS

Male Wistar rats of the A.R.C. strain of body weights 120 to 160 g. were maintained on a cube diet and tap-water. All rats were inoculated subcutaneously in the right flank with *Mycobacterium lepraemurium*.

The inoculum was prepared from a rat leproma which was finely minced in Dubos medium. Each rat was injected with 0.4 ml. of the suspension. The infected rats were then randomly distributed into three groups of 10; the first group was treated with intramuscular injections of cortisone acetate, the second with hydrocortisone acetate, the third group remained untreated. The doses were 2.5 mg. of steroid/rat, three times a week for the first month, twice a week for the next three months and then once a week until the end of the experiment. During this time each rat was weighed weekly. All rats were killed at six months after inoculation, and the lepromata were removed and weighed. The lepromata, spleens and livers of all the rats were examined histologically for the presence of acid fast organisms.

RESULTS

Effect of Steroid Treatment on Animal Growth.—The first obvious effect of the steroid treatment was the retardation of growth in the treated animals. Within 1 to 2 weeks after the commencement of the experiment, a diminution of growth rate was observed; this continued for the duration of the experiment. It appeared that hydrocortisone acetate was more potent in its growth-retarding effects than cortisone acetate.

Because of this effect, the dose of steroid was gradually reduced since it was feared that if the retardation of animal growth became too drastic it might interfere with the study of the effects of the steroids on the course of infection.

Fig. 1 shows the effect of adrenal steroid treatment on body weight. The mean body weights at weekly intervals of the control and of the treated groups are plotted for the duration of the experiment.

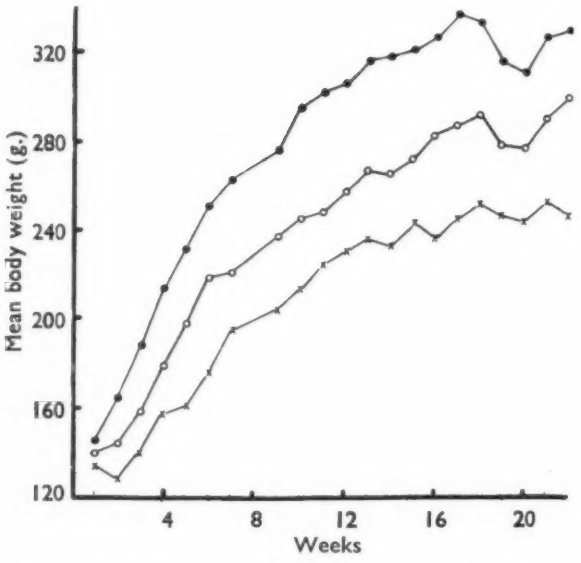

FIG. 1.—The effect of adrenal steroid treatment on body weight Each point represents the mean body weight of a group of 10 rats. The steriods were administered intramuscularly ; 2·5 mg. being given thrice weekly during the first month, 2·5 mg. twice weekly for the next three months, and then 2·5 mg. weekly until the end of the experiment. ●, Controls; O, cortisone acetate ; X, hydrocortisone acetate.

Effect of Steroid Treatment on the Growth of Lepromata.—Each of the rats infected with *Mycobacterium lepraemurium* subsequently developed a leproma at the original site of inoculation. As far as could be determined there was no significant difference in the time at which the lepromata appeared in the steroid treated and in the control groups. However, as the lepromata developed, it became apparent that they were very much smaller in the animals receiving steroids than in the untreated rats. Also several of the lepromata in the treated animals ulcerated and discharged before the end of the experiment, even though the lepromata were quite small when compared with those of the controls.

Six months after infection, the rats were killed and the lepromata removed. The lepromata were significantly smaller ($P<0.01$) in the rats receiving hydrocortisone acetate treatment, and in the rats treated with cortisone acetate ($P<0.001$). In this respect the effect of cortisone acetate was significantly more pronounced than that of hydrocortisone acetate ($P<0.01$). This difference in size was shown to be related only partially to the retardation of growth of the animals, since there is still a significant difference in weight ($P<0.01$) between the lepromata of the untreated and cortisone acetate treated animals when the results are expressed as g. of leproma/100 g. of body weight. These results are shown in Table I and Fig. 2.

TABLE I

THE EFFECT OF ADRENAL STEROID TREATMENT ON LEPROMA WEIGHT

The steroids were injected intramuscularly in doses of 2·5 mg./rat ; 3 times/week for the first month, twice a week for 3 months, and once a week until the end of the sixth month. The results of tests for significant differences between the mean weights of intact lepromata are shown.

Treatment	No. of Lepromata		Mean Weight of Intact Lepromata ± S.E.			
	Burst	Intact	g.	P	g./100 g. body wt.	P
Controls ..	1	9	13·46±2·14	—	4·04±0·68	—
Hydrocortisone acetate	4	6	5·94±0·86	<0·01	2·48±0·44	<0·1 >0·05
Cortisone acetate	3	6	2·44±0·70	<0·001	0·68±0·19	<0·01

Effect of Steroid Treatment on the Spread of Infection.—Smears and sections were made from the lepromata and spleens of all the rats, and sections only from the livers. There were innumerable acid fast organisms in all the lepromata, and as far as could be determined there was no appreciable difference in the distribution of organisms in the lepromata of either the treated or the untreated rats. However, the smears and sections of the spleens of the control animals showed a somewhat different picture from those of the treated rats. As shown in Table II, the number of animals showing acid fast bacilli in the spleen was smaller in the steroid treated groups than in the controls. This effect was most pronounced in the cortisone acetate treated group, in which only 1 out of 8 of the rats showed bacilli in the spleen. The results from the liver

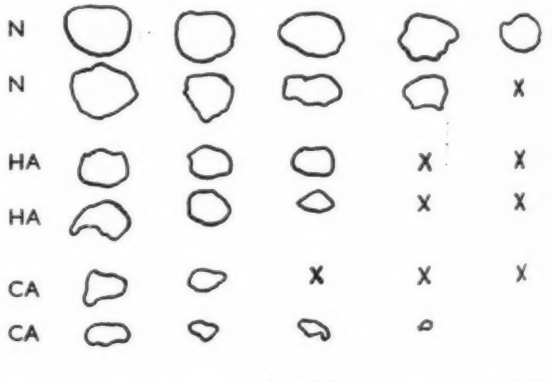

FIG. 2.—Lepromata from the normal untreated rats (N) and from the hydrocortisone acetate (HA) and cortisone acetate (CA) treated rats when removed 6 months after inoculation. The symbol X indicates lepromata which had burst and discharged before the end of the experiment. The scale is in cm.

sections were somewhat inconclusive, since only 4 rats from the control group of 10 animals showed acid fast bacilli.

TABLE II

NUMBER OF RATS SHOWING ACID FAST BACILLI IN LEPROMA, SPLEEN AND LIVER

Treatment	Smears		Sections	
	Leproma	Spleen	Spleen	Liver
Controls	10/10	9/10	7/10	4/10
Hydrocortisone acetate	7/7	6/10	3/10	4/10
Cortisone acetate	8/8	1/8	1/8	2/8

DISCUSSION

Prolonged administration of either cortisone or of hydrocortisone acetate does not increase the severity of the infection; indeed the smaller lepromata and the slight reduction in the spread of acid fast bacilli to the spleen in the steroid treated rats indicates that the reverse has taken place. The reduction in leproma size is not entirely related to the retardation of growth of the treated animals; it seems likely that the reduced leproma size is due mainly to the anti-inflammatory action of the steroids. The fact that cortisone acetate is more effective in this respect is not unexpected since other experiments performed in this laboratory have shown that cortisone acetate when injected intramuscularly into the rat is more potent than hydrocortisone acetate as an anti-inflammatory agent.

The results of this investigation are in agreement with the observations of Naguib and Robson (1956), who showed that prolonged administration of cortisone acetate in mice infected intracorneally with murine leprosy decreased the development of both macroscopic corneal and systemic lesions. However, these authors found that large numbers of acid fast bacilli were present in the livers and spleens, whereas in the present work the steroid treatment appeared to have reduced the spread of bacilli to these organs.

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Brit. J. Pharmacol. (1958), 13, 98.

A COMPARISON OF THE PITUITARY INHIBITORY EFFECTS OF PREDNISONE, PREDNISOLONE, AND HYDROCORTISONE

BY

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Prednisone, prednisolone and hydrocortisone, administered in doses of 1 mg. daily for thirty days, depressed the growth rate of rats. Prednisolone and hydrocortisone caused atrophy of the adrenal glands and increased the relative weights of the thyroid glands and testes. The apparently similar effects produced by prednisone were not significant. The seminal vesicles were not affected significantly by prolonged administration of prednisone, prednisolone or hydrocortisone. Pretreatment of rats with these steroids prevented the adrenal ascorbic acid depletion normally caused by laparotomy under ether anaesthesia. Prednisolone was slightly more potent and prednisone was considerably less potent than hydrocortisone in producing these effects.

It is well known that elevated blood levels of corticosteroids depress the secretion of adrenocorticotrophic hormone (corticotrophin, ACTH) by the anterior pituitary gland. This has been shown by many laboratory workers (Ingle and Kendall, 1937 ; Ingle, Higgins, and Kendall, 1938 ; Sayers and Sayers, 1947). Clinicians have also found that corticoids depress adrenocortical activity and that abrupt cessation of corticoid therapy may result in the signs of transient adrenal insufficiency. The development of prednisone (△'cortisone) and prednisolone (△'hydrocortisone) has provided clinical workers with corticosteroid-like substances more potent than cortisone and hydrocortisone and lacking the undesirable mineralocorticoid effects. Clinically these compounds also appear to depress the secretion of ACTH (Bunim, Black, Bollet, and Pechet, 1955 ; Pechet, 1955 ; Kupperman, Blatt, Vesell, Gagliani, Weisbader, and Vosburgh, 1955). Therefore, it was of interest to compare the pituitary inhibitory actions of these synthetic corticoid analogues with that of hydrocortisone. The experiments described in this paper were performed to study some effects of prolonged administration of these compounds and also to investigate their action on the secretion of ACTH which normally follows stress.

MATERIALS AND METHODS

The experiments were performed on male albino Wistar rats fed on a diet of cubes (diet 41, Lane-Petter and Dyer, 1952) and water and kept at a constant temperature of 70° F.

Prednisone (Merck), prednisolone (Merck) and hydrocortisone free alcohol (Glaxo) were suspended in normal saline. Lyophilized adrenocorticotrophic hormone (ACTH, Armour) was dissolved in normal saline to prepare solutions containing 1 International Unit/ml. which were kept at room temperature for not more than one hour. Solutions for injection were prepared immediately before use by diluting the 1 i.u./ml. solutions with normal saline.

Experimental Procedures. — (1) Fifty-one rats, weighing from 110 to 130 g., were divided into eight groups. Two groups served as controls, the rats in one group receiving no treatment and those in the other daily injections of normal saline only. Prednisone, prednisolone or hydrocortisone was administered to the remaining groups daily in doses of 0.1 or 1.0 mg. All the injections were made subcutaneously in volumes of 0.1 ml./rat. The animals were weighed frequently, and after 30 days' treatment they were killed with ether, and their adrenal glands, testes, seminal vesicles and thyroid glands were removed, dissected free from adhering tissue and weighed as quickly as possible to minimize the lost of moisture. (2) Four hundred and forty-eight rats, weighing from 120 to 160 g., received subcutaneously normal saline only or doses of 1.25 or 5 mg./100 g. body weight of a normal saline suspension of prednisone, prednisolone or hydrocortisone. The injections were given in volumes of 0.5 ml./100 g. The animals were anaesthetized with ether and mock adrenalectomized (Hodges, 1953) 1, 2, 4, 8, 16, and 32 hr. after receiving the injections. They were allowed to recover from

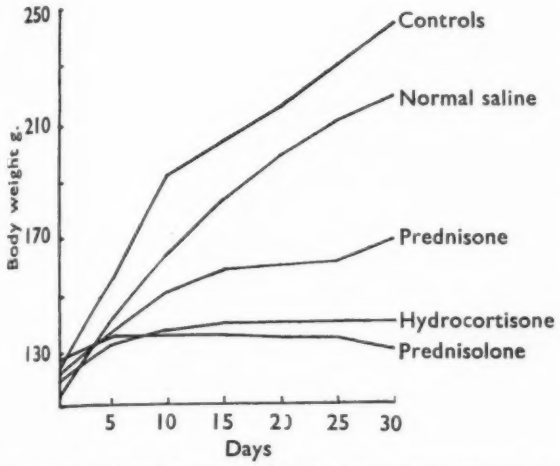

FIG. 1.—Growth rates of rats receiving daily subcutaneous injections of 1 mg. prednisone, prednisolone or hydrocortisone.

the operation and were killed 1 hr. later. Control rats were killed without any previous surgical interference. The adrenal glands were removed, dissected free from periadrenal tissue, weighed rapidly and transferred to 4% trichloracetic acid solution for estimation of their ascorbic acid contents by the method of Roe and Kuether (1943).

The animals were given access to food and water throughout the experimental periods.

RESULTS

Effects of Prolonged Administration of Prednisone, Prednisolone, and Hydrocortisone.—Doses of 0.1 mg./day/rat of the three steroids produced no significant differences between the growth rates of the treated animals and those of the controls and caused no morphological changes in the endocrine glands and accessory sex organs which were examined. However, a 10-fold increase in dosage produced marked changes in growth rate. Rats treated with prednisone gained weight less rapidly than the control animals. This effect was more marked with hydrocortisone, which completely arrested growth after it had been administered for about five days, and even greater with prednisolone, which caused some loss of weight after a similar period. These results are shown in Fig. 1. The steroid-treated rats were in poor condition and exhibited pronounced muscular weakness after about five days' treatment. Post-mortem examination of the animals showed that the steroids had caused adrenal atrophy (Table I). The effect of hydrocortisone was more marked than that of prednisolone. The effect of prednisone on adrenal weight was not significant. The weights of the testes and thyroid glands expressed in terms of body weight were significantly ($P<0.05$) greater in the animals which had received prednisolone or hydrocortisone than in the controls. Prednisone also increased the relative weights of the thyroid glands but did not increase significantly the testis weight. The apparent differences between the weights of the seminal vesicles of the treated groups and those of the controls were not significant. The *absolute* weights of these organs were in every case less than in the controls, but this is not surprising since prolonged steroid treatment inhibited the general growth rate so markedly.

Effect of Laparotomy on the Ascorbic Acid Concentration in the Adrenal Glands of Rats Pretreated with Prednisone, Prednisolone or Hydrocortisone.—Subcutaneous injections of normal saline, or saline suspensions of the steroids, resulted in no detectable changes in adrenal ascorbic acid concentration. Mock adrenalectomy under ether anaesthesia produced marked adrenal ascorbic acid depletion in the rats which had been pretreated with normal saline only. Previous treatment with prednisone, prednisolone or hydrocortisone diminished the fall in adrenal ascorbic acid concentration normally caused by the operation (Fig. 2). A dose of 1.25 mg. prednisolone

TABLE I

ADRENAL, THYROID, TESTIS AND SEMINAL VESICLE WEIGHTS IN RATS AFTER 30 DAYS' TREATMENT WITH PREDNISONE, PREDNISOLONE AND HYDROCORTISONE

The asterisk indicates that the difference from controls is statistically significant ($P<0.05$).

Treatment	No. of Animals	Adrenal Weight mg./100 g. Body Weight		Thyroid Weight mg./100 g. Body Weight	Testis Weight g./100 g. Body Weight		Seminal Vesicles g./100 g. Body Weight
		Left	Right		Left	Right	
Prednisone, 1 mg./day	6	6·1±0·5	6·1±0·7	8·1±0·2*	0·7±0·04	0·69±0·05	0·49±0·03
Prednisolone, 1 mg./day	6	5·6±0·4*	5·2±0·4*	10·9±0·4*	0·88±0·05*	0·92±0·05*	0·52±0·07
Hydrocortisone, 1 mg./day	6	4·6±0·4*	4·35±0·4*	9·6±0·7*	0·81±0·04*	0·84±0·04*	0·43±0·07
Saline, 0·1 ml./day	9	8·0±0·4	7·0±0·3	7·1±0·4	0·57±0·04	0·59±0·05	0·39±0·03
Uninjected controls	6	6·7±0·5	6·1±0·3	7·0±0·2	0·54±0·01	0·55±0·01	0·36±0·02

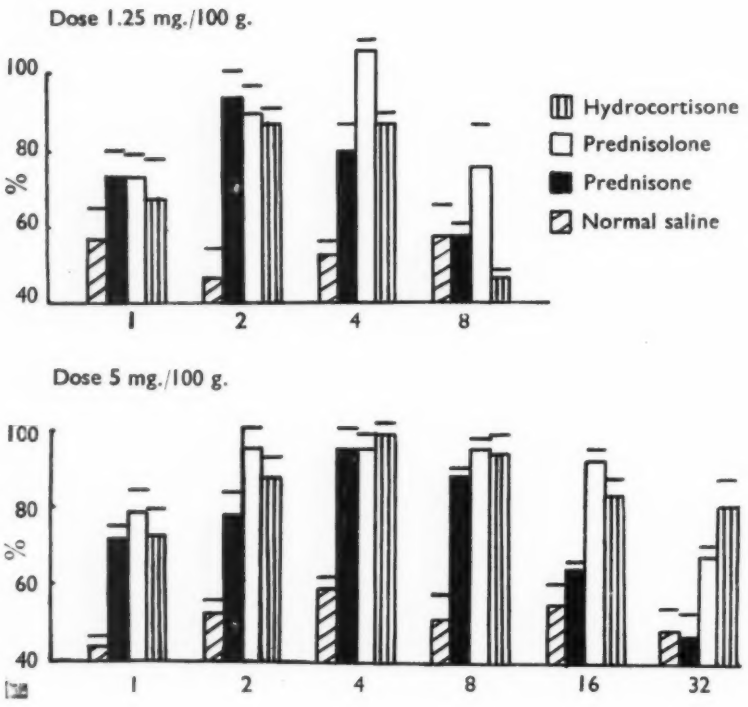

FIG. 2.—The effect of prednisone, prednisolone and hydrocortisone on the adrenal ascorbic acid depletion caused by mock adrenalectomy under ether anaesthesia. Ordinate: adrenal ascorbic acid concentration, 1 hr. after the operation, expressed as a % of the control level in unoperated rats. The numerals below the columns represent the time intervals in hours between the administration of the steroids and the performance of the operations. The lines above the columns represent the standard error.

prevented completely the adrenal ascorbic acid depletion only when the stress was applied 4 hr. after its administration. At other time intervals this dose of prednisolone was only partially effective. The same dose of prednisone or hydrocortisone was never more than partially effective. In a dose of 5 mg. prednisolone blocked completely the fall in adrenal ascorbic acid for a period of up to 16 hr. after its administration. The same dose of hydrocortisone produced a similar but less prolonged action. The action of prednisone was only transient and its effect in preventing adrenal ascorbic acid changes completely persisted for only a few hours.

It was probable that the steroids prevented the stress-induced depletion in adrenal ascorbic acid by interfering with the release of ACTH rather than by acting directly on the adrenal cortex. This was confirmed by the following experiment. Thirty rats weighing 120 to 150 g. were injected subcutaneously with 7.5 mg. hydrocortisone/100 g. and the animals were divided into five equal groups. One group served as controls. Four hours later the rats in the other four groups were anaesthetized with ether and injections were made into their exposed right femoral veins as follows: group (1) normal saline, group (2) heparinized blood from normal rats, group (3) heparinized blood from adrenalectomized rats 22 days after the removal of their adrenal glands, and group (4) normal saline containing 0.2 mu. ACTH/ml. All the intravenous injections were made in volumes of 3 ml./100 g. body weight. The animals were killed 1 hr. later and their adrenal ascorbic acid contents were determined. The mean adrenal ascorbic acid levels in each group were subtracted from the mean level in the control animals. The results are given in Table II. Normal saline and blood from normal rats produced no significant change in adrenal ascorbic

TABLE II

ADRENAL ASCORBIC ACID DEPLETION IN RATS

The rats were injected subcutaneously with 7·5 mg. hydrocortisone 4 hr. before and were killed 1 hr. after the intravenous injection of various fluids (3 ml./100 g. body weight). Adrenal ascorbic acid level in the controls was 524±11 mg./100 g. adrenal tissue.

Treatment	Adrenal Ascorbic Acid Depletion. mg./100 g. Adrenal Tissue. ± Standard Error
Normal saline	6±8
Heparinized blood from normal female rats	21±9
Heparinized blood from female rats 22 days after adrenalectomy	70±12
Normal saline containing 0·2 mu. ACTH/ml.	100±7

acid. However, ACTH and blood from rats adrenalectomized 22 days previously, which contains approximately 10 mu. ACTH/100 ml. (Cox and Hodges, 1958), produced significant adrenal ascorbic acid depletion.

Discussion

The results of experiments performed in many laboratories indicate that prolonged administration of corticoids causes marked adrenocortical atrophy and depresses the growth rate of rats (Ingle *et al.*, 1938; Ingle, 1941; Ingle and Meeks, 1952; Wells and Kendall, 1940; Winter, Silber, and Stoerk, 1950). Prednisolone produces a similar effect and the results described above indicate that it is more potent in inhibiting growth but less potent in causing adrenal atrophy than hydrocortisone. Prednisone is less potent than hydrocortisone in both respects. Prednisolone and hydrocortisone increased significantly the weights of the thyroid and testes relative to the total body weight. However, the body weights of the steroid treated animals, at the end of the experimental period, were considerably less than those of the controls. It can only be concluded that the growth of the thyroid glands and testes was not inhibited as markedly as was the general body growth of the animals.

There is little doubt that the adrenal cortical steroids depress the secretion of ACTH by the pituitary gland. The results of the experiments described in this paper indicate that prednisone and prednisolone produce a similar effect. However, in the rat, prednisolone is only slightly more potent than hydrocortisone and prednisone has a considerably weaker action in suppressing stress-induced ACTH secretion. It has been established that, in the rat as in other species, prednisone and prednisolone are considerably more active biologically in, for example, suppressing inflammation, maintaining life in adrenalectomized animals, depositing glycogen in the liver, promoting the uptake of [^{32}P] by adrenals, pituitary and thymus glands, etc., than the naturally occurring corticoids (Ducommun, Ducommun, and Baquiche, 1955; Donato, 1955). It appears, therefore, that the pituitary inhibitory activity of these compounds is not always related to their potencies in other respects. Thus it might be expected that the synthetic corticoid analogues could possess an additional advantage over cortisone and hydrocortisone when used therapeutically that they should not tend to depress endogenous ACTH production to such an extent. However, many clinical workers (Bunim *et al.*, 1955; Pechet, 1955; Kupperman *et al.*, 1955), using 17-ketosteroid excretion as an index of adrenocortical activity, have found that prednisone and prednisolone are considerably more active than cortisone and hydrocortisone in suppressing ACTH secretion in man.

Corticoids cause adrenocortical atrophy and prevent the adrenal ascorbic acid depletion normally caused by stress by interfering with the release of ACTH from the pituitary gland rather than by acting directly on the adrenal cortex. This was demonstrated by Ingle and Kendall (1937), who found that the atrophy of the adrenal cortex normally caused by the chronic administration of adrenocortical extracts could be prevented by the simultaneous administration of ACTH. Similar evidence was obtained by Sayers and Sayers (1947) and in the present work from the results of experiments which showed that ACTH still caused adrenal ascorbic acid depletion in rats pretreated with corticosteroids.

Since hydrocortisone and prednisolone were so effective in inhibiting ACTH release, the possibility was investigated of modifying the technique of Sayers, Sayers, and Woodbury (1948) for the bioassay of ACTH by substituting, for hypophysectomized animals, rats in which pituitary adrenocorticotrophic activity had been effectively blocked. Hodges (1955) described a method using rats previously treated with deoxycorticosterone acetate (DCA). The method suffered from the disadvantage that it lacked precision, and, owing to the sparingly soluble nature of DCA in arachis oil, large volumes of solution had to be injected subcutaneously to produce complete inhibition of ACTH release. The solution frequently tended to leak from the site of injection. The hydrocortisone or prednisolone treated rat appears to be more suitable and, at the present time, experiments are in progress using such animals for the estimation of ACTH in blood.

Our thanks are due to Merck and Co., Inc., for generous gifts of prednisone and prednisolone and to Glaxo Laboratories Ltd. for hydrocortisone. The expenses involved in the work were defrayed by a grant to one of us (J. R. H.) from the Medical Research Council.

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SOME PHARMACOLOGICAL PROPERTIES OF MUREXINE (UROCANOYLCHOLINE)

BY

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Murexine (urocanoylcholine, [2-β-imidazol-4(5)-ylacryloyloxyethyl] trimethylammonium bromide) has been shown to possess ganglion stimulating and neuromuscular blocking actions in cat, dog and rat. The blockade has been shown to be of the depolarizing type both on the basis of the resemblance of the pharmacological properties of the substance to that of known depolarizing blocking agents, and also directly by recording the depolarization produced in the endplate region of the gracilis muscle of the rat.

The recent appearance of several reports on the pharmacology of murexine (urocanoylcholine, [2-β-imidazol-4(5)-ylacryloyloxyethyl] trimethylammonium bromide) (Erspamer and Glässer, 1957; Grelis and Tabachnick, 1957; Quilliam, 1957) has prompted us to give a fuller account of some experiments carried out with this compound in 1955 which have so far been reported only in abstract form (Keyl and Whittaker, 1955).

The compound is of interest in being a naturally occurring choline ester. It was first identified by Erspamer and Benati (1953) in three Mediterranean species of whelks of the family Muricidae and later in three other North Atlantic Muricidae (Whittaker and Michaelson, 1954; Whittaker, 1955; Keyl, Michaelson, and Whittaker, 1957).

Our experiments confirm that murexine has a ganglion stimulating and neuromuscular blocking action. We have also demonstrated by means of the scanning electrode technique of Burns and Paton (1951) that, in the rat, the neuromuscular blockade is of the depolarizing type.

METHODS

Hydrolysis by Cholinesterases.—This was measured at 37° and *p*H 7.4 in bicarbonate buffer (0.023 M-$NaHCO_3$ in equilibrium with 95% N_2 and 5% CO_2 in the gas phase) using the Warburg manometric technique as modified by Ammon (1933). The following esterase preparations were used: purified bovine red cell cholinesterase (Winthrop Stearns); rat plasma; dog plasma; human plasma; Harvard human plasma fraction IV-6-3 (kindly supplied by Dr. D. M. Surgenor and stabilized by addition of 1% gum acacia to the medium). The rate of hydrolysis of murexine is expressed as μl. CO_2 evolved from the buffer/mg. dry wt. of enzyme preparation/hr. (Q_{UrCh}), or as % of the corresponding rate with acetylcholine (Q_{ACh}).

Animal Experiments.—The effect of intravenous administration of murexine bromide on blood pressure, respiration and neuromuscular transmission was determined on 17 dogs and 6 cats. The cats were anaesthetized with 0.6 ml./kg. Dial with urethane solution (Ciba) and the dogs with 0.7 ml./kg. Dial with urethane solution or 30 mg./kg. pentobarbitone sodium. Respiration was recorded on a smoked drum by means of a tambour; blood pressure was similarly recorded by means of a mercury manometer connected to a cannula in the femoral artery. Heparin was used as anticoagulant. The effect of the drug on neuromuscular transmission was determined by recording isotonic contractions of the gastrocnemius muscles in response to supramaximal electrical stimulation once every 2 sec. through the sciatic nerve. Rectangular wave stimuli of 0.1 m.sec. duration and 10 to 20 V. strength were delivered by a Grass model 3C stimulator through shielded platinum electrodes 4 mm. apart placed on the nerve distally to a crushed region.

Scanning Electrode Technique.—Muscle action potentials and surface depolarization in the endplate region of the gracilis muscle of the rat were measured by means of the scanning electrode technique of Burns and Paton (1951). Potentials were led through cathode followers into a D.C. amplifier and recorded photographically after display on a double beam cathode ray tube. To facilitate the interpretation of the record, the beam recording action potentials was made to move in an axis at right-angles to that recording the endplate potentials.

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RESULTS

Hydrolysis of Murexine by Cholinesterases

The rate of hydrolysis of murexine by representative mammalian cholinesterases is shown in Table I. It will be noted that the ester is split at a negligible rate by the acetocholinesterase of bovine red cells and by rat and dog plasma. It is split at approximately 7% of the acetylcholine rate by human plasma and by highly purified human plasma cholinesterase. The identity of the relative rates of hydrolysis of murexine by these last two preparations, the second representing a 1,600-fold purification of cholinesterase relative to the first, shows that the hydrolysis of murexine by human plasma is brought about by the cholinesterase and is not contributed to significantly by other plasma esterases. This conclusion is confirmed by the complete inhibition of hydrolysis obtained with 10 μM. eserine, an inhibitor and concentration specific for cholinesterases.

TABLE I

HYDROLYSIS OF MUREXINE BY CHOLINESTERASES

* Substrate concentration: red-cell preparation, 10 mM; others, 30 mM. † Substrate concentration: 10 mM. ‡ Calculated on assumption that the lyophilized preparation contains 3% enzyme preparation in NaCl-gelatine-phosphate stabilizer, as stated in Winthrop Stearns' specification.

Cholinesterase Preparation	Q_{ACh}*	Q_{UrCh}†	$\frac{Q_{UrCh}}{Q_{Ach}} \times 100$	% Inhibition by 10 μM. Eserine
Bovine red-cell (purified)	$7{\cdot}1 \times 10^4$‡	0	0	—
Rat plasma (unpurified)	6·4	0	0	—
Dog plasma (unpurified)	38	0	0	—
Human plasma (unpurified)	37	2·6	6·8	100
Human plasma fraction (IV-6-3)	$6{\cdot}2 \times 10^4$	4×10^3	7·0	—

TABLE II

EFFECT OF MUREXINE ON RESPIRATION, BLOOD PRESSURE AND NEUROMUSCULAR TRANSMISSION IN THE CAT AND DOG

In column (f) the numerals show the change in twitch height with the range in brackets, + denotes an increase and — a neuromuscular block.

Dose (μg./kg.) (a)	Respiration		Blood Pressure		Neuromuscular Transmission (% Change in Twitch Height), Dog (f)
	Cat (b)	Dog (c)	Cat (d)	Dog (e)	
10	—	—	None	None	—
30	None	Transient increase	—	—	—
50	None		None	Slight fall	None
100	None		None	Fall followed by slight rise	+9 (0 to +14)
200	Slight increase		Slight fall		+16 (+9 to +22)
300	—	Progressive increase	Slight rise	Progressive rise	−40 (−14 to −60)
500	Stimulation followed by failure		Definite rise		−80 (−52 to −88)
1,000	—	Stimulation followed by failure	—	Marked rise	−98 (−87 to −100)

Animal Experiments

The effect of murexine on the respiration and blood pressure in the cat and dog and the effect on neuromuscular transmission in the dog are summarized in Table II.

Effect on Respiration.—In the dog, murexine at a dose level of 30 μg./kg. produced a transient increase in respiration. Increasing doses up to and including 500 μg./kg. caused a progressively longer and more pronounced increase. At 1 mg./kg. murexine produced a short

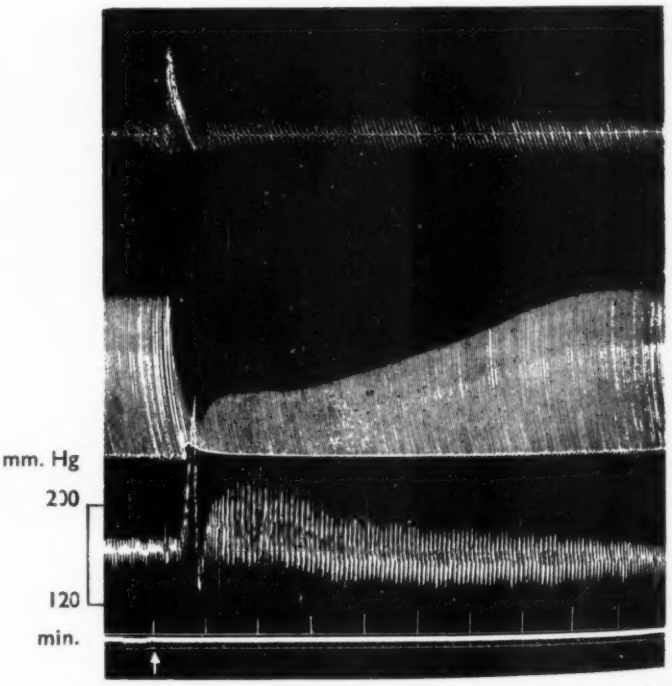

FIG. 1.—Effect of 0·55 mg./kg. of murexine injected intravenously at the arrow into the anaesthetized dog. Upper trace, respiration; middle trace, record of the isotonic twitch of the sciatic gastrocnemius muscle in the response to nerve stimulation; lower record, blood pressure.

stimulation followed by respiratory failure. Artificial respiration for 10 to 20 min. was always sufficient to save the animal. Respiratory failure was always manifest when the neuromuscular transmission, as indicated by the response of the sciatic-gastrocnemius preparation, was completely depressed, and was thus probably due to neuromuscular blockade in the respiratory muscles. Murexine in the cat produced the same effects, but a dose about seven times greater was needed to evoke effects than in the dog.

Blood Pressure.—A dose of 50 μg./kg. of murexine in the dog caused a slight depressor response. Increasing the dose up to 500 μg./kg. usually caused a depressor response followed by a pressor response of progressively greater amplitude and duration. The same effects were noted in the cat, but again the doses required were higher.

Neuromuscular Transmission. — Murexine caused no change in neuromuscular transmission in the dog in doses up to 100 μg./kg., while doses of 100 to 200 μg./kg. caused either no change or a slight increase in the twitch height of up to 20%. This effect might be due to an anticholinesterase action of the drug (Foldes, Erdös, Baart and Shanor, 1957). Above 200 μg./kg. blockade began to be manifest; this progressively increased with increasing doses and was 90 to 100% complete at 1 mg./kg. The type of neuromuscular blockade obtained with 550 μg./kg. of murexine in the dog is shown in Fig. 1, where the effects on respiration and blood pressure can also be seen.

The dose of murexine required to produce 90 to 100% neuromuscular blockade in three different mammalian species is presented graphically in Fig. 2. The corresponding doses of decamethonium are included for comparison. It will be seen that the species variations in the potency of the two drugs run parallel, the potency decreasing from the cat, through the dog, to the rat.

Effect of Tetraethylammonium (TEA) and Neostigmine.—TEA in a dose of 10 mg./kg. blocked the effect of murexine (500 μg./kg.) on blood pressure and neuromuscular transmission. The neuromuscular blocking action of murexine was not reversed by neostigmine in the dog or rat; indeed, in the rat, recovery was delayed by neostigmine.

Depolarizing Action.—It is well known that many neuromuscular blocking agents such as decamethonium and suxamethonium, exert their effect mainly by producing a prolonged depolarization of the endplate region. This is in contrast

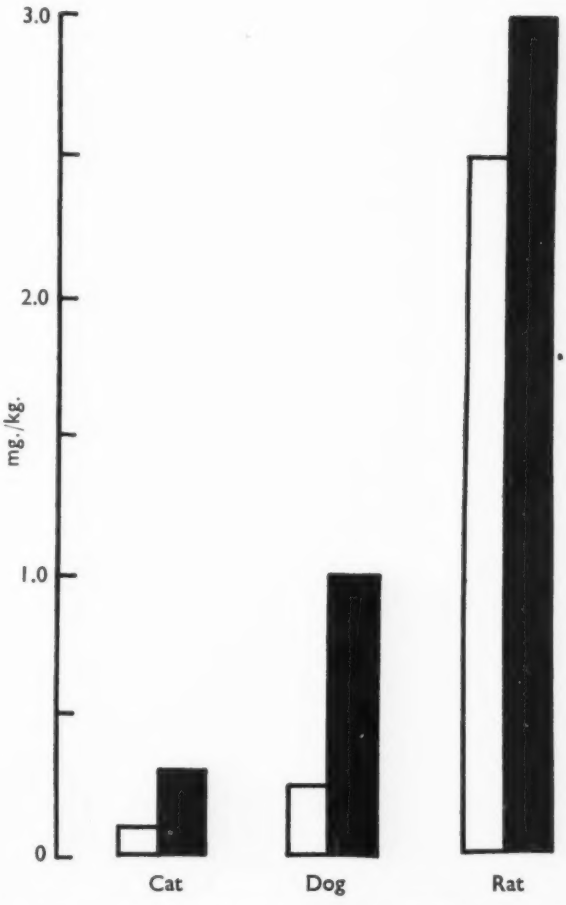

FIG. 2.—Species variation in the neuromuscular blocking action of murexine (solid columns) compared with that of decamethonium (open columns). Ordinates, dose of blocking agent required to produce 90 to 100% neuromuscular block.

to the type of blockade seen with tubocurarine, in which the drug blocks the depolarizing effect of acetylcholine without itself producing depolarization. It was of interest to find out if murexine had a direct depolarizing action on the endplate region, using the scanning electrode technique of Burns and Paton (1951). The arrangement of the electrodes and the potentials recorded are indicated in Fig. 3*a*. Fig. 3*b* shows that 40 sec. after the injection of the drug, the surface of the muscle in the endplate region became depolarized, as evinced by the negative potential recorded between a scanning electrode (*Sc*) and the stationary electrode (*St*) at the end of the muscle. Fig. 3*c* shows that the drug reduces the magnitude of the summed action potentials recorded by the scanning electrodes in response to periodic supramaximal stimulation of the motor nerve, showing that fewer

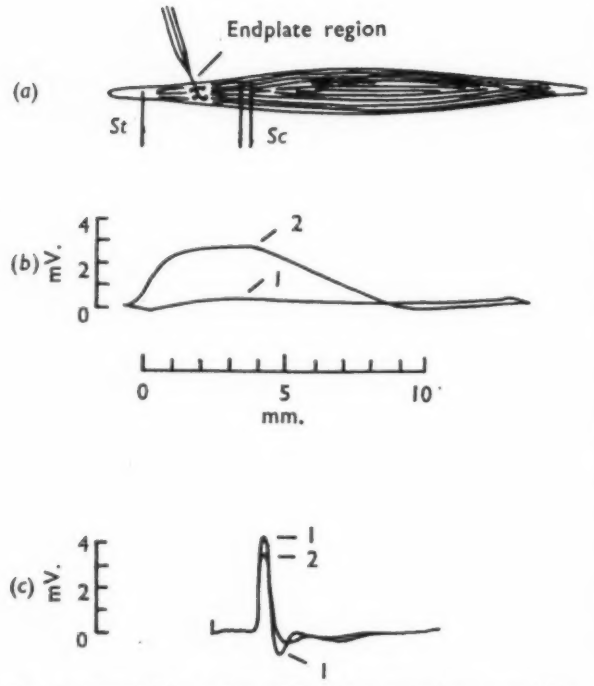

FIG. 3.—Depolarizing action of 3 mg. kg. murexine on endplate region of the gracilis muscle of the rat. (*a*) Diagram of electrodes used and potentials recorded. *St* is the stationary electrode and *Sc* the scanning electrodes. (*b*) Tracing of the potential of the scanning electrodes (*Sc*) relative to the stationary electrode (*St*) on the musculotendinous junction. Abscissa, distance of *Sc* from *St* in mm. (*c*) Tracings of the summed action potentials of the gracilis muscle under control conditions (1) and 40 sec. after injection of murexine (2). Negative potentials are shown as an upward deflexion of the traces. The potentials were recorded between the two scanning electrodes (*Sc*).

muscle fibres are responding to the nerve stimulation as the result of the blockade set up by the drug.

DISCUSSION

The results summarized above are in general agreement with those of other workers and show that murexine has both ganglion stimulating and neuromuscular blocking actions. The rise in blood pressure is most likely due to stimulation of sympathetic ganglia, since it was antagonized by the ganglion blocking agent TEA. Murexine is primarily a depolarizing type of neuromuscular blocking agent. This is shown by the spastic paralysis which it produces in birds (Erspamer and Glässer, 1957) in contrast to the flaccid paralysis produced by tubocurarine, the species variation in sensitivity to the drug, which closely parallels that of decamethonium, the inability of eserine to reverse the neuromuscular blocking action, and, finally, its depolarizing action on mammalian endplates as demonstrated by the scanning electrode experiments. The combination of ganglion stimulating and neuromuscular blocking action is also seen in other compounds, such as tetrakis (dimethylaminomethyl)methane tetramethobromide (Kensler, Langemann and Zirkle, 1954), and, interestingly enough, in β,β-dimethylacryloylcholine (Holmstedt and Whittaker, unpublished observation), the choline ester which occurs in place of murexine in the Muricidae *Thais floridana* (Whittaker, 1957 ; Keyl *et al.*, 1957).

The action of murexine was short-lasting in all three species examined. This cannot be attributed to hydrolysis by plasma esterases, since in two of these species, rat and dog, the rate of hydrolysis of the ester by plasma was negligible (Table I). In the analogous case of suxamethonium, where hydrolysis by plasma cholinesterase has been advanced as explanation for the short-lasting action of the drug, there is also no correlation between the rate of plasma hydrolysis and the duration of neuromuscular blockade in different species (Hansson, 1958).

The murexine bromide used in this work was kindly supplied by Dr. J. A. Aeschlimann, Hoffmann-La Roche Inc., Nutley, N.J. We are also most grateful to Dr. E. F. Van Maanen and Dr. W. L. Kuhn for assistance with the scanning electrode experiments. The work was supported by a grant [B-863 (C3)] from the U.S. Public Health Service.

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Brit. J. Pharmacol. (1958), **13**, 107.

A COMPARISON OF ANTIFIBRILLATORY DRUGS IN THE HEART-LUNG PREPARATION

BY

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Quinidine, amethocaine, Trasentin and atropine have been tested for their ability to stop atrial fibrillation produced in the heart-lung preparation of the dog by stimulating the right atrium in the presence of acetylcholine. The results suggest that atropine acts by specifically antagonizing acetylcholine, but that amethocaine and Trasentin act chiefly by virtue of their quinidine-like properties.

The method of producing atrial fibrillation in the heart-lung preparation of the dog, as described by Burn, Vaughan-Williams and Walker (1955), provides a preparation in which atrial fibrillation can be initiated and continued at will. It therefore offers suitable conditions for evaluating antifibrillatory drugs. However, this fibrillation requires the continuous infusion of acetylcholine and is abolished by atropine.

Few studies have been carried out in which antifibrillatory drugs have been tested directly for their efficacy in preventing or stopping experimentally induced atrial fibrillation. More frequently, a less direct technique has been used. Thus Dawes (1946) described a method of determining the maximum rate at which rabbit atria would respond to electrical stimuli, and suggested that drugs which reduced this rate might prove useful in treating atrial fibrillation in man.

We have investigated the relative activity of a series of drugs in abolishing atrial fibrillation in the dog heart-lung preparation. The drugs to be tested were chosen because they had very dissimilar relative potencies when tested on electrically stimulated rabbit atria (Dawes, 1946) and when tested for their action in inhibiting the effect of acetylcholine on the isolated rabbit atria (Elio, 1948). It was of interest to find out how far the ability of these substances to stop fibrillation maintained by infusion of acetylcholine was due to their atropine-like (anti-acetylcholine) action and how far it was related to their quinidine-like property as measured by Dawes.

METHODS

Fibrillation in the Heart-lung Preparation.—The preparation was set up and atrial fibrillation was established as described by Burn *et al.* (1955). The minimal rate of infusion of acetylcholine at which fibrillation would persist for at least 15 min. after stopping the stimulation was determined. This rate was continued for the rest of the experiment.

The drug to be tested was dissolved in 0.9% NaCl solution and infused from a burette into the blood flowing into the superior vena cava. As soon as fibrillation was abolished the drug was stopped and the amount infused was recorded. Attempts were then made to re-establish fibrillation by electrical stimulation in the usual way. If it was re-established and persisted for more than 1 min. after the end of stimulation the drug was again infused until normal rhythm returned. The preparation was tested once more to see if fibrillation would persist. The final reading was therefore the least amount of drug required to abolish fibrillation and to prevent its being effectively re-established.

The presence or absence of atrial fibrillation was determined from the blood pressure record, from the electrocardiogram and by direct observation.

Quinidine-like Action on Rabbit Atria.—The method described by Dawes (1946) was followed exactly.

RESULTS

The results of nine experiments on the heart-lung preparation are set out in Table I.

DISCUSSION

By the method described here, it is not possible to control all the variable factors which might affect the amount of drug required to abolish fibrillation. For instance, the amounts of acetylcholine necessary to maintain fibrillation ranged from 1.0 to 12.0 mg./min., and it is possible that preparations differing in sensitivity to acetylcholine might also vary either directly or inversely

TABLE I

AMOUNTS OF DRUGS REQUIRED TO STOP ATRIAL FIBRILLATION

Expt.	Rate of Infusion of Acetylcholine (mg./min.)	Drug	Duration of Infusion of Drug (min.)	Amount of Drug Given (mg.)	Mean Amount (mg.)
1	3·2	Quinidine	10	10·0	13·9
2	1·2	,,	17	12·7	
3	1·0	,,	20	19	
4	4·0	Amethocaine	20	14·0	16·6
5	12·0	,,	16	19·2	
6	1·5	Trasentin	36	5·9	4·8
7	2·8	,,	9	3·8	
8	1·2	Atropine	2	0·005	0·005
9	1·2	,,	5	0·005	

in their sensitivity to antifibrillatory drugs. However, the results do not give evidence of any consistent variation of this sort. There is, for instance, a threefold difference in sensitivity to acetylcholine between the two experiments with amethocaine, and the less sensitive preparation required slightly more amethocaine to abolish fibrillation. On the other hand, the results from the experiments with quinidine and Trasentin suggest that those preparations which require most acetylcholine to produce fibrillation require least antifibrillatory drug to abolish fibrillation.

When comparing antifibrillatory potencies it would be desirable to infuse the drugs at such a rate that effective doses were given in equal times. During prolonged infusions, some of the drug might be absorbed by tissues other than the heart or even metabolically inactivated in some way; in either case it would be necessary to give more of the drug at low rates of infusion than at higher rates. Comparison of the doses of drugs required and the durations of the infusions do not suggest that these complications have arisen in these experiments. Indeed, a heart-lung circuit allows only limited possibilities for elimination of added drugs.

The relative potencies of quinidine, amethocaine, Trasentin and atropine in abolishing atrial fibrillation are given in Table II and compared with their relative potencies in reducing the maximum rate at which isolated rabbit atria can be driven (Dawes, 1946) and in antagonizing the action of acetylcholine on isolated rabbit atria (Elio, 1948). It is evident that atropine is very effective in the preparations involving the use of acetylcholine. The ratios of activity of quinidine to atropine in antagonizing acetylcholine and abolishing fibrillation are approximately the same, and it would seem at first sight that quinidine exerted its antifibrillatory action here solely by virtue of its atropine-like activity. However, this cannot be true of Trasentin, which has 1/10 of the activity of atropine in antagonizing acetylcholine, but only 1/1500 of its activity in abolishing fibrillation. Furthermore, since amethocaine is quite devoid of atropine-like activity on atria, its antifibrillatory activity must be due to a "quinidine-like" property. Though on the preparation of Dawes amethocaine has 11.5 times the activity of quinidine, it has only 1/8 the potency of quinidine in abolishing atrial fibrillation induced by acetylcholine. This discrepancy might be due to the fact that it actually potentiates the action of acetylcholine on the atria.

TABLE II

RELATIVE ACTIVITY OF QUINIDINE, AMETHOCAINE, TRASENTIN AND ATROPINE IN VARIOUS PREPARATIONS

The asterisk indicates a value taken from our own results. The numerals in the table are the ratios of activity, on a molar basis, taking quinidine = 1.

Preparation	Quinidine	Amethocaine	Trasentin	Atropine
Driven rabbit atria (Dawes, 1946)	1	11·5	0·59	0·17*
Antagonism of acetylcholine on rabbit atria (Elio, 1948)	1	Potentiation	400	4,000
Abolition of atrial fibrillation in the dog heart-lung preparation	1	0·13	1·6	2,470

We are grateful to Mr. H. W. Ling and Mr. R. Smith for assistance with these experiments.

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CHRONIC TOXICITY OF DEXTRAN SULPHATE IN RABBITS

BY

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(RECEIVED MAY 25, 1957)

The chronic toxicities of two samples of low molecular dextran sulphate from different sources were studied in rabbits. Both samples caused retardation in weight gain, cachexia and general osteoporosis with spontaneous fractures and epiphyseolyses. Heparin-treated controls were used to exclude the possibility that the toxic effects could be connected with prolonged anticoagulant treatment. It is concluded that prolonged administration of dextran sulphate causes systemic toxic effects which are not related to its anticoagulant activity.

Grönwall, Ingelman, and Mosiman (1945) showed that the toxicity of dextran sulphate declined with decreasing molecular weight. Later Walton (1954) investigated the toxicity of dextran sulphates with still lower molecular weights and found that " compounds with an average molecular weight of about 7,500 show qualitatively similar anticoagulant action to that of heparin and comparable low toxicity."

However, the chronic toxicity of such compounds has not been definitely established. In 1952 we began with investigating the effects of prolonged administration of low molecular dextran sulphate to rabbits. We regularly obtained a typical syndrome consisting of increasing weakness, spontaneous fractures of the bones and/or paresis of the legs. The doses used were 10 to 50 mg./kg. administered intravenously three to six times weekly. The syndrome described was seen in practically all animals if the treatment was extended over a sufficient period of time which varied between 4 and 12 weeks and depended on the compound and dose used. With heparin, no such symptoms could be produced with doses up to 50 mg./kg. under similar conditions.

The purpose of the present work was to describe the results of a typical experiment with low molecular dextran sulphate prepared in our laboratories and to ascertain if a sample of commercially available dextran sulphate produced for clinical use showed qualitatively similar effects.

In the two experiments recorded in this paper we used heparin-treated animals as controls to eliminate the possibility that the anticoagulant effect *per se* might influence the results. The doses used were chosen on the basis of our earlier experiments. No attempt was made to correlate the dosage with the doses used clinically for anticoagulant treatment.

MATERIALS AND METHODS

Dextran Sulphates.—In the first experiment a compound synthesized in our laboratories by Ingelman and Mårtensson was used. This preparation had an intrinsic viscosity of 0.042 at 25°. Using the light scattering method, the average molecular weight was determined by K. Granath and found to be about 3,000. The sulphur and nitrogen contents were 17.5% and less than 0.001% respectively. The substance will be referred to as dextran sulphate I. It was employed as a 1% sterile solution of the sodium salt in physiological saline, prepared immediately before use. No buffering agents were used.

In the second experiment, a British commercial dextran sulphate (Dexulate, Glaxo, Batch No. 2) was used. This preparation contained 1,000 dextran sulphate units/ml. and 1% buffering agents. It will be referred to as dextran sulphate II. [One international dextran sulphate unit is approximately equivalent in anticoagulant activity to one international unit of heparin (Ricketts, Walton, van Leuven, Birbeck, Brown, Kennedy, and Burt, 1953).]

Heparin.—In both experiments a Swedish heparin (Vitrum Lot A-54), sodium salt, containing 100 i.u./mg. was used. It was freshly prepared as a 1% sterile solution in physiological saline before use.

Experimental Animals.—Albino rabbits of the same breed and of both sexes and about five to six months old were used. The animals were housed in individual cages at room temperature. The food consisted of grain mixture, clover hay, beets and carrots. Food and water were unrestricted.

Plan of Experiments.—The drugs were administered intravenously. The animals were weighed at least once a week and examined regularly. The doses were not adjusted to animal weight changes during the course of treatment.

Experiment I.—Three groups of rabbits were injected once daily except on Saturdays and Sundays with dextran sulphate I, heparin or saline. According to earlier experience, the dose of dextran sulphate producing definite changes within four to eight weeks was found to be about 20 mg./kg. As we intended to estimate the lowest toxic dose, we started with a dose of 10 mg./kg. and later raised the doses of both dextran sulphate and heparin to 20 mg./kg.

Experiment II.—A group of five rabbits was injected once daily with dextran sulphate II. Another group of five rabbits served as controls and were injected with doses of heparin equipotent in anticoagulant activity. As we had no previous experience with dextran sulphate II and wished to produce definite changes within a reasonable time of treatment, the doses used were approximately 500 dextran sulphate units/kg. and 500 i.u. of heparin respectively. These doses corresponded to about 45 mg./kg. of dextran sulphate II and 5 mg./kg. of heparin.

Results

Experiment I.—As can be seen from Fig. 1, the dextran sulphate I group shows only slight retardation in weight gain during the first seven weeks of treatment during which 10 mg./kg. daily of dextran sulphate I was administered. The average weight gain was 0.47 kg. in the dextran sulphate group I as compared to 0.67 kg. and 0.71 kg. in the heparin and saline groups respectively.

After increasing the doses of both drugs to 20 mg./kg. daily, signs of toxicity became apparent within two weeks in the dextran sulphate group. The animals lost weight and showed gradually increasing weakness and cachexia while the control groups remained in good condition. The growth rate in the heparin group seemed to be slightly depressed as compared to the saline group.

The rabbits in the dextran sulphate I group developed paresis of the hind legs and/or spontaneous fractures. According to earlier experience, animals with such symptoms die within a few days if the treatment is continued. Therefore, these animals were killed with pentobarbitone sodium given intravenously at times indicated in Fig. 1.

At autopsy, the only gross lesions found were those in the skeletal system. Signs of increased fragility of the bones were observed in all animals of the dextran sulphate I group. Further characteristic lesions were seen near the large joints in form of epiphyseolyses and deformation of articular surfaces. Two of the

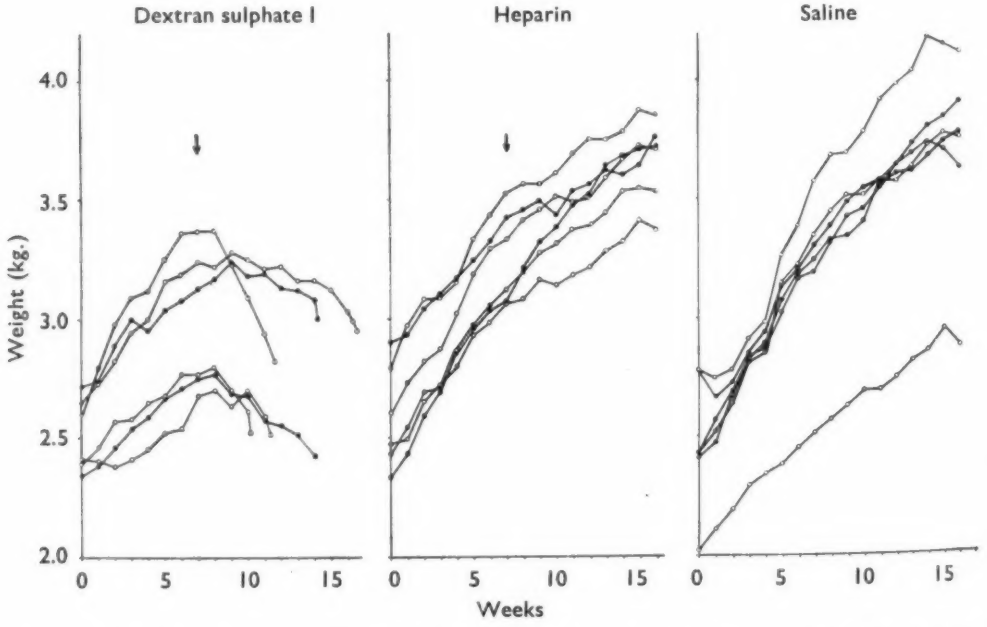

FIG. 1.—Growth curves for rabbits treated with dextran sulphate I, heparin and saline respectively. The first values represent the weight at the start of treatment. The arrows indicate the day when dosage was raised from 10 mg./kg. to 20 mg./kg. for both drugs. The injections were given i.v. daily except on Saturdays and Sundays. The last values represent the weight on the day on which the animals were killed.

rabbits had fractures of the humeral diaphysis. Five out of six rabbits in this group showed epiphyseolyses of the femur, tibio-fibula or humerus. In two animals fractures of the lumbar vertebrae were found.

Haematomas were seen only in connexion with fractures. Small haematomas were found typically around loosened parts of the compacta, the latter remaining attached to muscular insertions. Apparently, the pareses observed may be explained by the occurrence of fractures of the vertebrae and/or loosened muscular insertions as well as by epiphyseolyses.

Post mortem, *x*-ray examination of the skeleton revealed considerable thinning of the compacta, rarefaction of the spongiosa and numerous fractures in all dextran sulphate I treated animals. The rarefaction of the spongiosa was pronounced in the epiphyses of the long bones, in the pelvis and the lumbar vertebrae.

No gross lesions were found in the heparin and saline groups. The histological findings of experiments I and II will be described together.

Experiment II.—As can be seen from Fig. 2 all rabbits receiving dextran sulphate II showed retardation in weight gain. During the first three weeks of treatment this group had an average weight gain of 0.21 as compared to 0.53 kg. in the heparin group. After the third week, the dextran sulphate II group exhibited marked weight loss, while the heparin group showed a further average weight gain of 0.27 kg. during the second three weeks.

On physical examination, no signs for external or internal bleedings which primarily could be explained by decreased coagulability of the blood could be observed. From the fourth week of treatment, all animals in the dextran sulphate II group showed increasing weakness, spontaneous fractures of the bones and/or paresis of the legs. One of the animals died after the sixtieth injection. The others were killed with pentobarbitone sodium at times indicated in Fig. 2 because of pareses and spontaneous fractures.

At autopsy gross lesions identical to those described in experiment I were found. Three out of five rabbits in this group showed multiple epiphyseolyses of the humerus or femur. Two of the rabbits had fractures of the femoral diaphysis.

As in experiment I, post mortem *x*-ray examination of the skeleton disclosed considerable thinning of the compacta and rarefaction of the spongiosa. To illustrate the degree of osteoporosis, the thickness of the compacta in the femoral diaphyses was measured from roentgenograms magnified twenty times. The average thickness of the femoral compacta measured at corresponding sites was found to be 0.9 mm. in the dextran sulphate II group and 1.3 mm. in the heparin group.

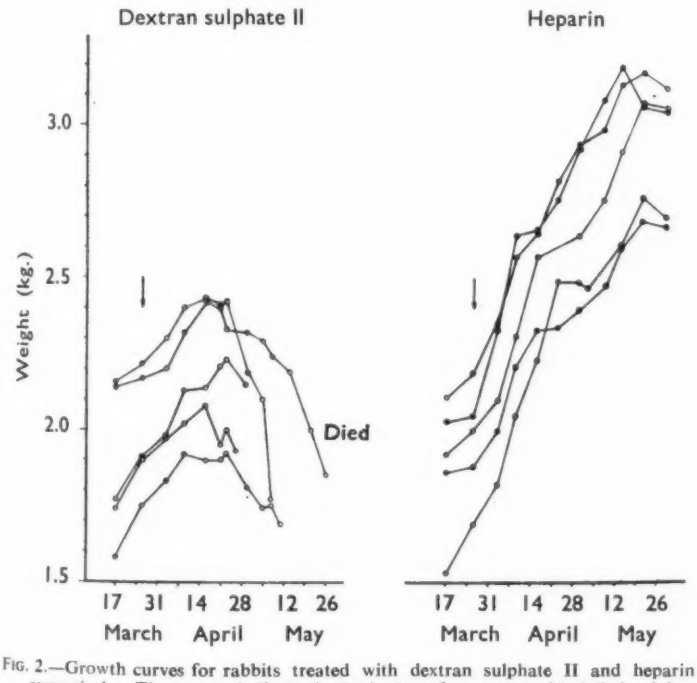

FIG. 2.—Growth curves for rabbits treated with dextran sulphate II and heparin respectively. The arrows indicate the beginning of treatment with daily i.v. injections of 500 i.u. of dextran sulphate and with 500 i.u. of heparin respectively. The last values represent the weight on the day on which the animals were killed.

Histopathological Findings.—No constant histopathological findings were found in the liver, kidneys, spleen, and adrenals of the animals treated with dextran sulphates when compared with the controls. The histopathological study of decalcified slices from costochondral junctions showed generalized bone absorption. The number of osteoblasts was diminished while osteoclasts were present in great numbers. The bone trabeculae in the growth zone were thin and diminished in number. They often appeared devoid of the osteoblastic layer, the latter being replaced by an almost continuous seam of osteoclasts.

In the arteries of the lungs, another typical lesion was seen fairly constantly. The lumina of

the arteries were often wholly or partially occluded with fat and/or haemopoietic tissue and occasionally contained small fragments of bone or cartilage. These lesions are probably embolic in nature and secondary to osteoporosis and fractures.

Discussion

Walton (1954) concluded that the toxicity of large molecular dextran sulphates depends on the interaction of these compounds with plasma proteins and the formed elements of the blood. The substances used by us are of such a low molecular size that, according to the results of Walton (1954), no interaction with blood components can be expected. Therefore the findings described in this paper appear to have a different pathogenesis.

The location of the principal lesions in the skeleton and especially in the epiphyseal growth zones suggests that dextran sulphate may be deposited at these sites. However, such deposition has as yet not been demonstrated.

The interpretation of the pathogenesis of the osteoporosis described lies beyond the scope of this work, but as a working hypothesis we assume that dextran sulphate might act by competition with naturally occurring sulphated polysaccharides in bone and cartilage and in this way might interfere with normal bone metabolism.

It is evident from the growth curves that the growth rate was depressed long before spontaneous fractures occurred. Therefore, the cachexia observed is not necessarily the result of osteoporosis or of trauma caused by fractures. Possibly, the cachexia may indicate more general systemic effects not demonstrable anatomically.

In spite of the fact that, in experiment I, 1,000 to 2,000 i.u./kg. of heparin was used, no comparable chronic toxic effects were noticed. It is therefore concluded that dextran sulphate exhibits systemic toxic effects which are not related to its anticoagulant activity.

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VASOACTIVE SUBSTANCES IN THE NASAL MUCOSA

BY

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(RECEIVED AUGUST 19, 1957)

Besides histamine another smooth muscle stimulating substance has been found in the nasal mucosa of dog and sheep. It is an acid which is readily oxidized by neutral aqueous potassium permanganate and osmium tetroxide, and is thermostable at *p*H 10·8 but not at *p*H 2·8. The purification of this acid has been followed by using the atropinized tortoise intestine as a test object, which is insensitive to acetylcholine, 5-hydroxytryptamine and small amounts of histamine. Both the tortoise intestine and rat colon are contracted by this acidic substance. The amount of histamine found in sheep nasal mucosa ranged from 12 to 23 μg./g. tissue and in dog nasal mucosa from 6 to 13 μg./g. tissue.

The presence of large numbers of blood vessels in the nasal mucous membrane lends the mucosa readily to engorgement and oedema as in allergic vasomotor rhinitis. The factors which control vasomotor tone in the nasal mucosa have not been extensively investigated. Of the various vasoactive substances known to be present in animal tissues only histamine has so far been reported to occur in the nasal mucosa (Buhrmester and Wenner, 1936) and in nasal secretions of allergic rhinitis (Troescher, Ancona, and Kerr, 1945). The experience of one of us (C.C.T.) that the antihistaminic drugs are not completely effective in controlling the nasal congestion of allergic rhinitis has prompted us to investigate the possibility that other pharmacologically active substances besides histamine may be present in the nasal mucosa. This paper reports the finding of an unidentified vasoactive substance besides histamine in extracts of the nasal mucosa.

METHODS

Collection of Nasal Mucosa.—The heads of sheep obtained from the abattoir or of dogs which had been anaesthetized with pentobarbitone and bled to death were used. The heads were split sagittally and the mucosa was stripped from the nasal septum and turbinates. The mucosa was rapidly washed in running tap water, dried between filter paper, weighed and extracted.

Acid Extraction.—Nasal mucosa was minced with 3 ml. 0.1 N-HCl/g. tissue and the mixture briefly heated to boiling point. The extract was neutralized, centrifuged to remove insoluble residue and the supernatant solution used.

Phosphate Extraction.—Nasal mucosa was minced with 1 ml. M/15 phosphate buffer (*p*H 7.6)/g. tissue. The mixture was alternately frozen and thawed 3 times and centrifuged. The residue was re-extracted with an equal volume of phosphate buffer and the two extracts pooled.

Acetone Extraction.—Nasal mucosa was minced with 4 ml. acetone/g. tissue and the mixture left in the refrigerator overnight. The acetone extract was filtered off and the residue was re-extracted with an equal volume of 80% (v/v) acetone for another 3 hr. The acetone extracts were pooled and the acetone removed by evaporating *in vacuo*. The aqueous residue was used. For paper chromatography the aqueous solution whose *p*H was 5.6 to 6.3 was extracted 3 times with an equal volume of *n*-butanol previously saturated with water. The butanol extracts were pooled and evaporated to dryness *in vacuo*. The residue was taken up in ethanol and applied to filter paper for chromatographic development.

Paper Chromatography. — Chromatography was carried out by the ascending method at 25°. The solvent systems used were: Butanol-ammonia (100:1). 1 ml. ammonia solution (sp. gr. 0.910) was added to 100 ml. *N*-butanol. Water was then added to the solution until it became just saturated.

Butanol-acetic acid (100:1). 1 ml. glacial acetic acid was dissolved in 100 ml. *N*-butanol and water added to the solution until it became just saturated.

Areas were cut out from the chromatograms and eluted with M/100-sodium bicarbonate solution, using 1 or 1.5 ml. solution/10 cm.^{2} of paper. The elution was carried out with constant shaking for 1 hr.

Pre-treatment of Filter Paper.—Paper was treated with M/10-ammonium bicarbonate or M/10-am-

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monium acetate by passing the paper through the solution, removing the surplus fluid by pressing between blotting papers and hanging the filter paper up to dry.

Spray Reagents.—After spraying a chromatogram with neutral aqueous 1% $KMnO_4$ the chromatogram was allowed to dry in the air until the pink colour of the $KMnO_4$ had disappeared and the chromatogram had turned brown. This was important as traces of $KMnO_4$ contracted the tortoise intestine.

Treatment of a chromatogram with osmium tetroxide vapour was done in a closed vessel for 1.5 hr., after which the chromatogram was hung up in air for 1 hr. to remove traces of the reagent.

A 0.1% ninhydrin solution in chloroform was used. After the chromatogram had been sprayed with the reagent it was heated at 95° to 105° for 5 min.

The nitrous acid used was a solution of 1% $NaNO_2$ in 0.1 N-HCl.

Paper Electrophoresis.—Electrophoresis was carried out on Whatman filter paper No. 1 stretched horizontally in a closed chamber. The buffers used for electrophoresis were 0.033 M phosphate buffers (*p*H 8.5 and *p*H 5.3) and 0.05 M-glycine in HCl buffer (*p*H 3.5).

Isolated Tortoise Jejunum. — The land tortoise (*Cyclemys amboinensis*) was used. It was killed by pithing. The plastron was removed and the animal bled by removing the heart. A piece of jejunum was dissected out and suspended in a 20 ml. bath containing fluid of the following composition: NaCl 6.5 g., KCl 0.3 g., $CaCl_2$ 0.02 g., $NaHCO_3$ 0.10 g., glucose 0.50 g., water to 1,000 ml. The fluid was designed to prevent spontaneous contractions of the tortoise intestine so that it could be used for quantitative assays. Oxygen was used to aerate the medium. When first placed in the bath the gut was strongly contracted, but if the recording lever to which the preparation was suspended was weighted so as to keep the intestine under a tension of about 5 g. it soon relaxed, relaxation being aided by frequent changes of bath fluid. The weight of the lever was finally adjusted so as to keep the gut under a tension of 2 to 3 g. A loop of intestine which had been removed from the tortoise could be used the next day if the gut was covered with the solution described above and stored in the refrigerator at 10°. Substances tested on the tortoise intestine were kept in the bath for 45 to 60 sec. and were added to the bath at 4 to 5 min. intervals. Some pieces of intestine relaxed only slowly after contracting. In these instances the time interval between addition of substances to the bath was suitably increased.

Isolated Rat Colon.—The preparation used was similar to that described by Dalgliesh, Toh, and Work (1952).

Histamine Assay.—Histamine was assayed on the isolated guinea-pig ileum suspended in a 20 ml. bath containing Tyrode solution and 0.2 μg. atropine sulphate.

RESULTS

Histamine in Nasal Mucosa

The amounts of histamine found in sheep and dog mucosa are given in Table I. In the sheep the amount of histamine ranged from 12 to 23 μg./g. tissue while in the dog it varied from 6 to 13 μg./g. In four experiments on sheep tissue and four experiments on dog tissue the amounts of histamine extractable by acid and phosphate were compared. The values obtained by phosphate extraction were of the same order as those obtained by acid extraction, although they tended to be slightly lower.

TABLE I

HISTAMINE IN NASAL MUCOSA

Animal	μg. Histamine/g. Tissue	
	Acid Extraction	Phosphate Extraction
Sheep 1	12	11
,, 2	14	13
,, 3	16	12
,, 4	19	14
,, 5	19	—
,, 6	18	—
,, 7	23	—
Dog 1	7	6
,, 2	10	7·5
,, 3	13	10
,, 4	6	7

The Presence of an Unidentified Smooth Muscle-stimulating Substance in Extracts of Nasal Mucosa

Evidence that there was present in extracts of dog and sheep nasal mucosa a smooth muscle-stimulating substance which was not acetylcholine, histamine, or 5-hydroxytryptamine was obtained when the extracts were tested on the isolated rat colon and the isolated tortoise intestine.

Tortoise Jejunum.—The isolated tortoise jejunum is little stimulated by histamine and 5-hydroxytryptamine. Relatively large amounts of histamine (1 to 10 μg.) were needed to contract the tortoise gut in a 20 ml. bath. The effect of histamine was to produce a slow contraction of the gut, which could not be readily abolished by the antihistamine compounds mepyramine and promethazine without at the same time depressing the contractions obtained with acetylcholine. 5-Hydroxytryptamine in amounts of 0.2 to 20 μg. inhibited the contractions of the tortoise intestine to acetylcholine (Fig. 1). Although the amounts of 5-hydroxytryptamine tested varied 100 times, the inhibition due to 20 μg. 5-hydroxytryptamine was not much greater than that due to 0.2 μg. The tortoise intestine did not contract to 0.2 or

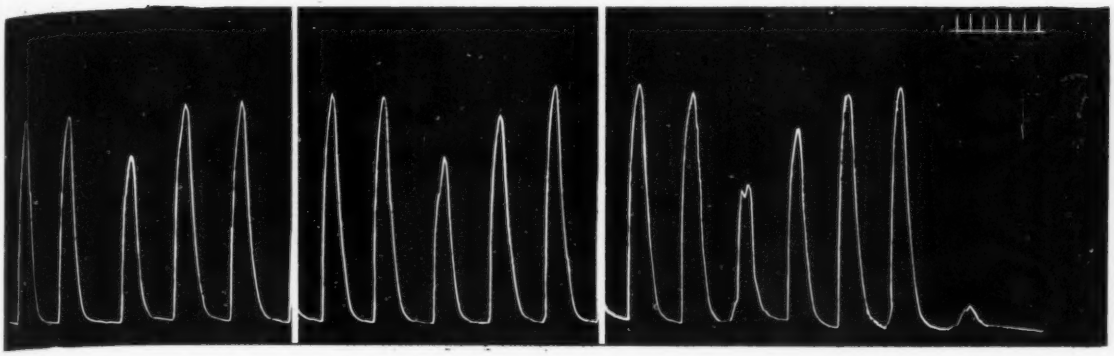

FIG. 1.—Inhibition by 5-hydroxytryptamine of the contractions of tortoise jejunum to 0·6 μg. acetylcholine. The acetylcholine was allowed to act on the gut for 45 sec. and repeated at 4 min. intervals. 5-Hydroxytryptamine was added to the bath 1 min. before the addition of acetylcholine and left in the bath during the period of action of acetylcholine as indicated. The amounts of 5-hydroxytryptamine tested were (*a*) 0·2 μg., (*b*) 2 μg., and (*c*) 20 μg. The gut did not contract to 0·2 μg. or 2 μg. but contracted slightly to 20 μg. as shown in (*c*) **Se.** Time: 1 min.

2.0 μg. of 5-hydroxytryptamine, but was slightly stimulated by 20 μg. Thus large amounts of 5-hydroxytryptamine had a weakly excitatory as well as an inhibitory effect on the gut. If the tortoise intestine is atropinized, it is not stimulated by acetylcholine, 5-hydroxytryptamine, or small amounts of histamine.

When acid, phosphate, or acetone extracts of nasal mucosa were tested on such an atropinized tortoise intestine it was found that the extracts caused the gut to contract strongly. In Fig. 2, X shows the contraction produced by a phosphate extract of 50 mg. sheep nasal mucosa. The effect of the extract was much greater than that produced by 0.75 μg. histamine, which was the amount present in this quantity of extract. In Fig. 2, Y indicates the contraction of the tortoise intestine to an acid extract of 50 mg. sheep nasal mucosa, which had previously been found to contain 1.0 μg. histamine. The stimulating effect of the extract, however, could not be accounted for by the histamine as the contraction due to 1.0 μg. histamine was again very much less than that due to the extract. The effect of the extracts on the tortoise intestine could not therefore be due to histamine, and since the gut was atropinized it could not be caused by acetylcholine. The effect could not have been caused by 5-hydroxytryptamine which, as already shown, had little or no stimulating effect on the tortoise intestine.

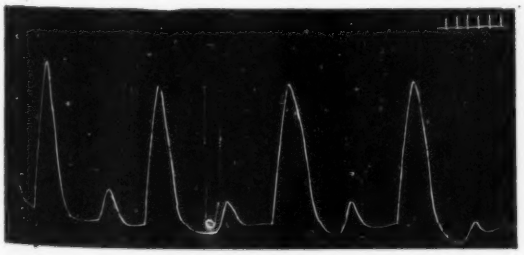

FIG. 2.—Contractions of isolated tortoise jejunum to (X) phosphate extract of 50 mg. sheep nasal mucosa, (Y) acid extract of 50 mg. sheep nasal mucosa and (Hi) histamine in the amounts indicated. The phosphate extract contained 15 μg. and the acid extract 20 μg. histamine/g. tissue. The bath held 20 ml. and contained 0·2 μg. atropine. Time: 1 min.

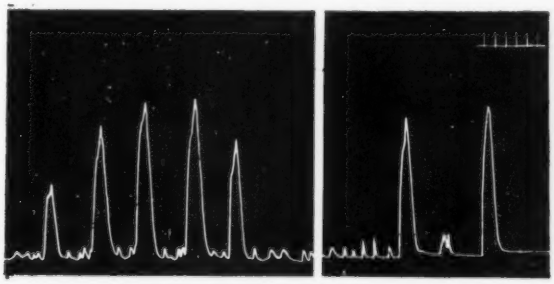

FIG. 3 —Effect of (X) acid extract of 100 mg. and 200 mg. sheep nasal mucosa and (Se) 5-hydroxytryptamine on isolated rat colon in 20 ml. bath containing 0·2 μg. atropine. In (*b*) the bath fluid contained 4 μg. BOL-148 as well as atropine. Time: 1 min.

Rat Colon.—Fig. 3 shows the effect of an acid extract of sheep nasal mucosa on the atropinized rat colon. In Fig. 3(*a*) the contraction produced by 200 mg. mucosa was matched by 0.02 μg. 5-hydroxytryptamine. In Fig. 3(*b*) the bath in which the gut was suspended contained D-2-bromo-lysergic acid diethylamide (BOL 148) as well as atropine. The effects of 0.02 μg. and 0.04 μg. 5-hydroxytryptamine were now abolished, but the contraction due to the extract remained. The effect could not therefore be due to 5-hydroxytryptamine or acetylcholine, nor could it be due to histamine, to which the colon is naturally insensitive.

Characteristics of the Unidentified Active Substance in Extracts of Nasal Mucosa

Dialysability.—The active substance could be dialysed from the crude extract into distilled water. However, even after 1.5 hr. of dialysis the greater part of the activity remained in the dialysing bag.

Effect of Trypsin and Chymotrypsin.—There was no loss of activity on incubating an extract of nasal mucosa at *p*H 8.0 with trypsin powder (crude preparation, British Drug Houses) or with crystalline chymotrypsin (Light). Both enzymes were used in a concentration of 10 mg./ml. of extract of nasal mucosa for 2 hr. at 39°. The active substance in the extract is unlikely to be a polypeptide as it was not destroyed by these enzymes.

Effect of Temperature.—An acetone extract of sheep nasal mucosa was adjusted to different *p*H and heated in a boiling water bath for 15 min. At the end of this period the samples were neutralized and their activity on the tortoise intestine tested. No loss of activity occurred at *p*H 10.8; there was a loss of 10% activity at *p*H 6.4 and 25% at *p*H 2.8.

Location of the Active Principle on Paper Chromatograms.—In order to separate the active principle on paper chromatograms 20 to 25 g. sheep nasal mucosa was extracted with acetone and the active principle taken up in *N*-butanol as described under "Methods." About ½ of the butanol extract was applied along a 32 cm. line on Whatman No. 1 filter paper for ascending chromatography. The position of the active principle on the chromatogram was located by dividing the chromatogram into zones and testing the eluates from these zones on the atropinized tortoise intestine.

On paper treated with 0.1 M-ammonium bicarbonate and using *N*-butanol-ammonia as developing solvent it was found that the active principle could be eluted from a zone lying between R_F 0.4 and 0.7. On paper treated with 0.1 M-ammonium acetate and using *N*-butanol-acetic acid as developing solvent it was found that the active principle lay in a zone between R_F 0.7 and 1.0.

If parallel strips from these chromatograms were sprayed with neutral aqueous potassium permanganate (1%) or exposed to osmium tetroxide vapour, no activity was present in the eluates of such treated chromatograms. On the other hand, spraying the chromatograms with ninhydrin, nitrous acid, or Pauly's reagent did not destroy the active principle. These results suggested that the active principle was a readily oxidizable substance but that it was not likely to be a base or a phenol.

Experiments were also made to purify larger quantities of extract on untreated Whatman No. 3MM paper using butanol-ammonia as developing solvent. The zone lying between R_F 0.4 and 0.7 was eluted with sodium bicarbonate solution and the eluate evaporated to dryness *in vacuo*. The residue obtained in this way was a mixture of sodium carbonate and purified extract. It was taken up in 0.9% NaCl solution for tests on the tortoise intestine. In one experiment (Fig. 4) the atropinized tortoise intestine was contracted by 0.5 to 1.0 mg. of solid.

Paper Electrophoresis.—In order to study the electrophoretic behaviour of the active principle

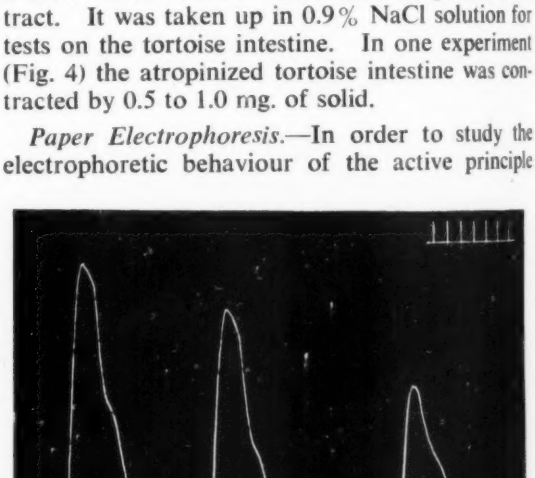

FIG. 4.—Contractions of the atropinized tortoise intestine to 1·0, 0·75 and 0·5 mg. of solids obtained from paper chromatograms of sheep nasal mucosa. The chromatograms were developed with butanol-ammonia and the zone lying between R_F 0·4 and 0·7 was eluted. Time: 1 min.

a certain degree of purification was necessary as it was found that crude extracts which contain lipid material do not show any movement on either acid or alkali buffered paper under a potential gradient of 12 V./cm. The active principle was therefore first isolated on paper chromatograms as described above, either using butanol-ammonia or butanol-acetic acid as developing solvent. In each case about 12 g. sheep nasal mucosa was used. The eluate from the chromatogram which contained the active substance was evaporated to dryness *in vacuo* over concentrated sulphuric acid. The residue was taken up in 0.2 ml. distilled water and about 60 μl. applied along a 4 cm. line drawn across a strip of Whatman No. 1 paper which had previously been soaked in buffer and equilibrated with the current turned on for 1 hr. Electrophoresis was then carried on for 4 hr. At the end of the experiment the paper strip was dried and divided into strips, 1.5 cm. long and 4 cm. wide. The position of the active principle after electrophoresis could therefore be located by testing the activity of each strip. This was done by immersing it for 2 min. in the bath in which the tortoise gut was suspended.

At *p*H 8.5 and under a potential gradient of 12 V./cm. the active substance was located on the anodic side of the origin. Using material purified on a butanol-ammonia chromatogram the active substance was found to have migrated 4.2 cm. from the origin towards the anode after 4 hr. On the other hand, with material obtained from a butanol-acetic acid chromatogram, the movement was only 1.0 cm. from the origin towards the anode. This smaller movement could be due to interference by lipid material which was not separated from the active substance on a butanol-acetic acid chromatogram. Endosmosis was in the direction of the cathode as shown by glucose, but no correction in the migration values has been made for this effect.

Using material purified on a butanol-ammonia chromatogram it was found that at *p*H 5.3 under a potential gradient of 12 V./cm. there was no movement of the active substance from the origin. At *p*H 3.5 the active substance was located 1.2 cm. from the origin towards the cathode after 4 hr. This movement, however, can be explained by endosmosis, because glucose was found to have migrated 2 cm. from the origin towards the cathode.

The tendency of the active principle to migrate towards the anode at alkaline *p*H and its lack of movement at acid *p*H suggested that it was probably an acid substance.

Discussion

The experiments described above show that there are two pharmacologically active substances present in the nasal mucosa. One of these is histamine and the other is an acid which is as yet unidentified. Neutral potassium permanganate and osmium tetroxide are used as reagents to detect unsaturated bonds, and as these compounds have been shown to destroy the activity of the acidic substance on the tortoise gut it would suggest that the acid is probably an unsaturated acid. The presence of unsaturated fatty acids in the lipids of olfactory mucosa has in fact been found from biochemical analyses by Heusghem and Gerebtzoff (1953). It has not yet been possible to identify the pharmacologically active acid of the nasal mucosa with Darmstoff (Vogt, 1957b), Irin (Ambache, 1957), the unsaturated G acid of human plasma (Gabr, 1956) or the unsaturated fatty acid of envenomed egg yolk (Vogt, 1957a), all of which stimulate smooth muscle.

The presence of another smooth muscle stimulating substance besides histamine in the nasal mucosa lends interest to the problem of allergic rhinitis. It may be that these substances are released in this condition to produce vascular engorgement and subsequent oedema.

We wish to thank Dr. A. Fanchamps of Sandoz, Ltd., Basle, for a gift of BOL-148.

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A QUANTITATIVE METHOD FOR THE ASSAY OF INHIBITORS OF ACID GASTRIC SECRETION IN THE RAT

BY

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Assays of inhibitors of gastric secretion are described by a method of recording continuously acid gastric secretion in the rat. A number of doses of inhibitor comprising several assay blocks can be administered in one preparation and complete assays with known fiducial limits performed with only two rats. Assays were carried out with vasopressin against secretion induced by histamine, and with atropine, methanthelinium and atropine methylnitrate against secretion induced by methacholine.

Methods for the quantitative assay of inhibitors of gastric secretion have been described for the rat and the dog. Visscher, Seay, Tazelaar, Veldkamp, and Vander Brook (1954) and Shea (1956) have described assay methods in the pylorus-ligated rat in which the reduction of spontaneous secretion due to an inhibitory drug is measured. Each rat receives only one dose of inhibitor, but by combining several experiments quantitative results can be obtained. In the pouch dog an essentially similar principle is used (Benjamin, Rosiere, and Grossman, 1950). A submaximal secretion (Code, Hightower, and Hallenbeck, 1951) is induced by an intravenous infusion of histamine, and this is reduced by the inhibitory drug. Although in this assay the same animal can be used repeatedly, only one dose of inhibitor is administered in each test so that a number of experiments must be combined to give a quantitative result.

The present method, which is based on the procedure for continuous recording of gastric secretion described in a previous paper (Ghosh and Schild, 1958), differs from the earlier methods in two main respects. (i) Several doses of inhibitor are administered in succession so that each experiment comprises one or more assay blocks. (ii) The inhibitory drug is given before the stimulant and is thus used to prevent rather than counteract the effect of the latter. In this respect the method resembles the "double histamine" test. The administration of several doses of inhibitor in the same preparation introduces certain complications arising from persistence of the effect of the inhibitor, but it eliminates sources of error due to animal variation and renders the assay considerably more efficient than previous methods in terms of numbers of animals used.

Two inhibitors were studied, vasopressin and atropine. Posterior pituitary hormones are known to inhibit gastric secretion (Dodds, Hills, Noble, and Williams, 1935; Gray, Culmer, Wells, and Wieczorowski, 1941; Wolf and Wolff, 1947) probably by their vasoconstrictor action (Cutting, Dodds, Noble, and Williams, 1937), and we have found vasopressin to be an effective inhibitor of histamine secretion in rats. The inhibitory effect of vasopressin is readily reversible so that in an assay there is relatively little interference from one dose to the next. The effects of atropine and related compounds, tested against methacholine stimulation, are less readily reversible and tend to be cumulative. This difficulty can be partly overcome by the use of a design in which the dose order is taken into account as recently shown by Rocha e Silva and Rothschild (1956): two atropine-like compounds, methanthelinium (Banthine) and atropine methylnitrate (Eumydrin) were assayed against atropine and it was possible to perform a statistically controlled assay with only two rats for each comparison.

METHODS

The method of Ghosh and Schild (1958) for the continuous recording of acid gastric secretion in the rat was used. Briefly, this method is based on perfusion of the stomach of the rat anaesthetized with

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urethane with a dilute sodium hydroxide solution and graphical registration of the *p*H of the emerging fluid. All drugs were administered intravenously, usually by single injections, in a volume of 0.2 to 0.4 ml. Antagonists were usually injected immediately before the agonists. The following drugs were used: histamine acid phosphate (activity expressed in terms of base), vasopressin (Pitressin), atropine sulphate, atropine methylnitrate (Eumydrin), and methanthelinium (Banthine, Searle).

RESULTS

Effect of Vasopressin on Acid Secretion Induced by Histamine

When an intravenous injection of vasopressin is administered together with or immediately before an intravenous injection of histamine, the normal secretory effect of histamine is inhibited. Fig. 1 shows (on the original circular chart) the

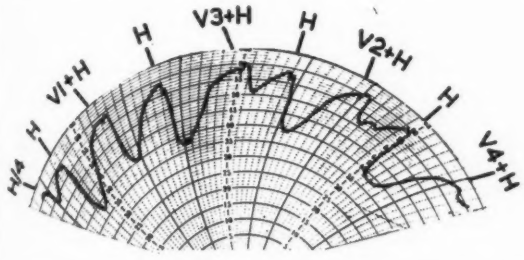

FIG. 1.—Effect of intravenous injections of 200 μg. histamine (H) and 200 μg. histamine with vasopressin (V_1=120 mU., V_2=170 mU., V_3=340 mU., V_4=480 mU.), on *p*H of stomach perfusate. Original circular chart. Distance between horizontal lines=0·5 *p*H units, between solid vertical lines=1 hr. In this and subsequent figures the ordinate represents the *p*H of the stomach perfusate following intravenous injection of drugs in the rat under urethane at 30°.

effect of four doses of vasopressin injected together with histamine, each dose of inhibitor being followed by a dose of histamine alone. The inhibitory effect of vasopressin is graded according to dose. A graded effect could generally be obtained within a four-fold dose range. In Fig. 2*a* the responses from individual experiments are joined, and in Fig. 2*b* linear regression lines are fitted to each set of experimental points; with one exception, the effect increases with dose and the slopes of the regression lines are approximately parallel.

Quantitative Assays with Vasopressin

These assays were planned as 2 + 2 assays with known concentrations of vasopressin. One or more blocks of four doses were administered in the same rat. At the beginning of each experiment, one or two control injections of histamine were given followed by combined injections of a constant dose of histamine with varying doses of vasopressin.

Three experiments with several doses of vasopressin in the same preparation were carried out. In one preparation three randomized blocks of four doses of vasopressin were administered and in the others two blocks of four doses. One of the assays with two blocks is shown in Fig. 3. A dose of 50 μg. histamine was combined with 14, 20, 42, and 60 mU. vasopressin and the experiment was treated as an assay of two solutions of vasopressin. Fig. 4 shows the regression lines for "standard" and "unknown." The activity ratio estimated from these results is 1.32 as against a true ratio of 1.43.

Analysis of variance of this experiment showed that: (i) the *mean square* for *regression* is significant at the 5% level though not at the 1% level; (ii) there is no evidence of deviation from parallelism; (iii) there is no significant difference between the two assay blocks. In the other two experiments similar results were obtained. The *mean*

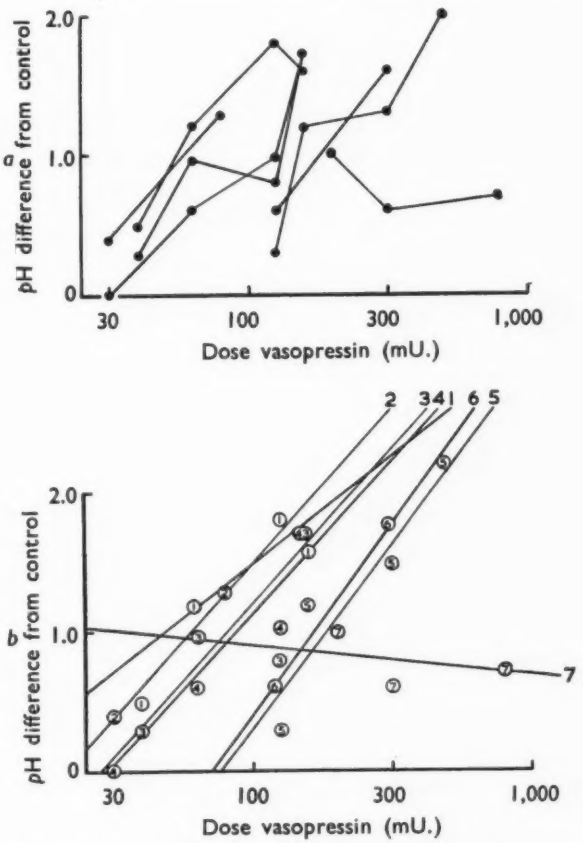

FIG. 2.—Dose/effect curves for vasopressin. The ordinate represents reduction of secretory effect by histamine+vasopressin as compared with histamine alone. In the upper panel responses from individual experiments are joined. In the lower panel calculated regression lines were fitted. Numerals in circles correspond to regression lines.

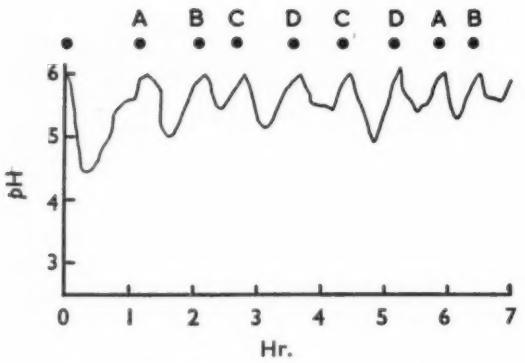

FIG. 3.—Two blocks of 4 doses of vasopressin injected together with a constant dose of histamine (50 μg.). A=20 mU.; B=60 mU.; C=14 mU.; D=42 mU. vasopressin.

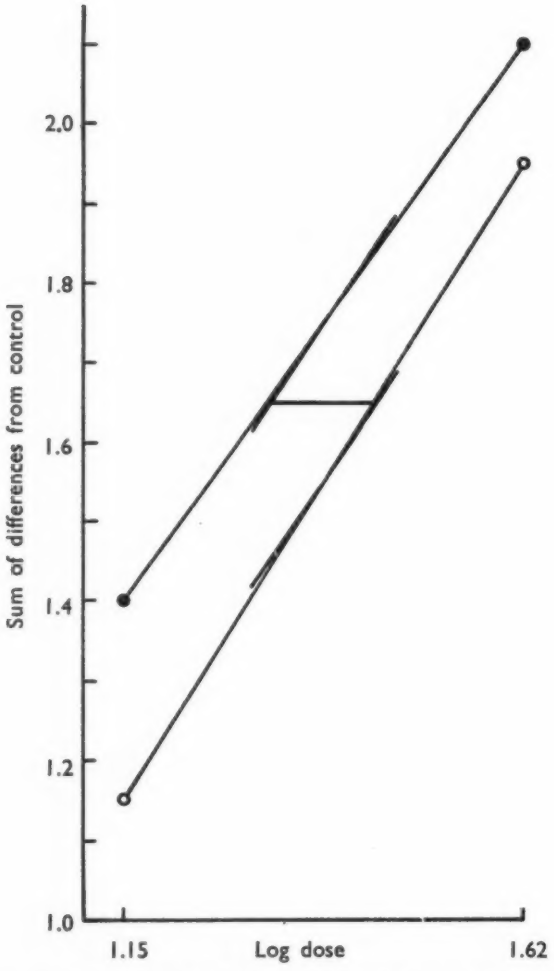

FIG. 4.—Regression lines fitted to vasopressin assay of Fig. 3.

square for *regression* was significant at $P<0.05$ in one assay and at $P<0.01$ in the other. There was no significant deviation from parallelism and no significant difference between successive assay blocks. In another series a dose of histamine was interpolated between doses of vasopressin to allow for more complete recovery from inhibition. This series involved three rats, each of which received four doses of vasopressin, the combined results being treated as a single assay. In the analysis of variance regression was highly significant; there was no evidence of deviation from parallelism. Differences between assay blocks were near-significant. Since each set of four doses was administered to a different animal, differences between assay blocks were to be expected. In conclusion, then, all the assays gave a satisfactory regression and no deviation from parallelism. The true and estimated activity ratios and the values for λ in the four assays are shown in Table I. The λ value expresses the

TABLE I

TRUE AND ESTIMATED ACTIVITIES AND λ VALUES OF FOUR ASSAYS WITH HISTAMINE AND VASOPRESSIN

Expt. No.	% Activities		λ
	True	Estimated	
194 (without recovery dose) ..	125	137	0·26
195 ,, ,, ,, ..	143	132	0·16
197 ,, ,, ,, ..	130	121	0·16
201, 202, 203 (with recovery dose) ..	140	224	0·11

intrinsic precision of the experiment. All assays gave reasonable λ values: the lowest value (highest precision) was obtained in the preparations which were allowed to recover between doses of inhibitor.

Effect of Atropine-like Substances on Methacholine-stimulated Secretion

Three experimental designs were employed to assay atropine-like substances against methacholine: 1. Continuous infusion of methacholine and inhibition by intermittent doses of atropine. When methacholine was administered by slow constant infusion in doses of 10 to 40 μg./hr., it produced a continuous stimulation of acid secretion which could be counteracted by injections of atropine. Fig. 5 shows the effects of intravenous injections of doses of atropine ranging from 0.03 to 0.3 μg. in a preparation stimulated by constant infusion of methacholine. Although graded effects could be obtained in this way with atropine the responses were irregular, and after large doses

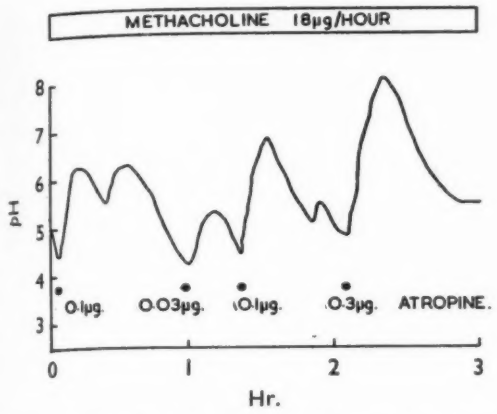

FIG. 5.—Inhibition of acid secretion by single intravenous doses of atropine superimposed on a slow intravenous infusion of methacholine.

the curve failed to return to the base line. Furthermore, continuous intravenous injection of methacholine produced considerable bronchial secretion, dyspnoea, and other toxic effects. 2. Intermittent injections of methacholine and atropine; dose-ratio assay. Single intravenous injections of methacholine produce a transient secretory response graded according to dose (Ghosh and Schild, 1958). Atropine reduces this response and the antagonism can be measured in terms of the dose of antagonist required to reduce the effect of a double dose of methacholine to that of a single dose. (This corresponds to a pA_2 determination, except that the antagonist is measured as a dose instead of as a concentration.) An experiment of this kind is shown in Fig. 6. Methacholine (2.5 μg.) produced a deflexion of about 1 *p*H unit, whereas 5 μg. produced a larger deflexion. Atropine (0.2 μg.) caused a slight reduction of the effect of 5 μg. methacholine and 0.4 μg. a larger reduction. By graphical extrapolation, the dose of atropine which reduced the effect of methacholine to that of half the dose was estimated as 0.47 μg. Table II shows other estimates of this quantity with methacholine and carbachol used as a stimulant. The activity of homatropine tested in this way was about 20 times less than that of atropine. 3. Intermittent injections of methacholine and two atropine-like substances. In these experiments constant doses of methacholine were combined with varying doses of atropine-like drugs. The experiments were carried out as 2 + 2 assays in a Latin square design. In one assay, consisting of a comparison of methanthelinium and atropine by a 4 × 4 Latin square design, only two rats were used, each receiving two blocks of four doses. Within each block consecutive doses of antagonist were administered, but recovery doses of methacholine were interpolated between the blocks. The results of this assay are shown in Table III and one of the two experiments is illustrated in Fig. 7. The ratio between large and small doses of inhibitor was three, and each time the larger dose produced a greater inhibition than the smaller dose. The analysis of variance of this assay (Table IV) gave highly significant F values for regression and for difference between rats. The F value for *dose order* does not quite reach the 5% level, but it is sufficiently large to suggest that position in the block is important. Fig. 8 shows that the regression lines for the two antagonists are parallel. The activity ratio for methanthelinium and atropine was 10.7 with 5% fiducial limits of 8.2 and 14.1.

TABLE II
DOSE OF ANTAGONIST REQUIRED TO REDUCE EFFECT OF AGONIST TO THAT OF HALF THE DOSE

Stimulant	Antagonist	Antagonist Dose (μg.)
Methacholine	Atropine	0·76
,,	,,	0·47
Carbachol	,,	0·72
Methacholine	Homatropine	12·5

TABLE III
RESULTS OF THE 2+2 ASSAY OF METHANTHELINIUM AND ATROPINE

Drug	Dose (μg.)	Maximum Deflexion of *p*H in Different Groups				Total
Atropine ..	1·0	1·4	0·9	1·9	1·9	6·1
	3·0	0·3	0·25	1·1	0·45	2·1
Methanthelinium	0·1	0·9	0·8	2·35	1·8	5·85
	0·3	0·3	0·2	0·55	0·7	1·75
Sum of groups ..		2·9	2·15	5·9	4·85	15·8

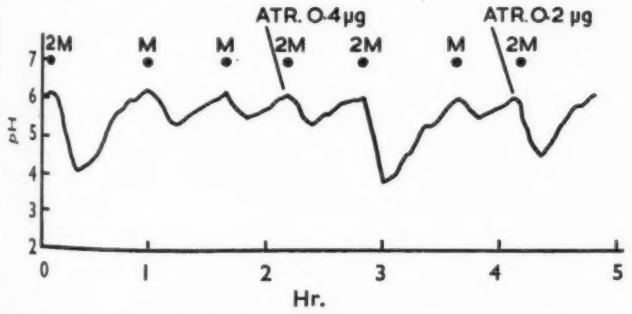

FIG. 6.—Inhibition of methacholine-induced secretion by simultaneous injection of graded doses of atropine.

This result is almost the opposite of that reported by Benjamin *et al.* (1950), who estimated the activity ratio of methanthelinium and atropine against histamine secretion in the dog (Table VII). In view of this discrepancy the comparison was repeated, this time with one rat for each block and with doses of methacholine after each dose of inhibitor. This assay gave an activity ratio of 10.7 (fiducial limits 4.4 to 29.1 at 5% level), thus confirming the previous results. The λ values and fiducial limits in this and the previous assays are shown in Table V. The λ value of the second methanthelinium assay was considerably larger than that of the first, suggesting that the more time-consuming method involving recovery doses had no special advantages in this instance. In another assay a comparison of atropine methylnitrate and atropine was carried out. This assay involved two rats, one of which

TABLE IV

ANALYSIS OF VARIANCE OF THE RESULTS OF THE ASSAY OF METHANTHELINIUM AND ATROPINE

Source of Variation	Sum of Squares	d.f.	Mean Square	F	
Between rats	2	1	2	50	$P<0.01$
Between groups	0·25	2	0·12	3	$P>0.05$
Dose order	0·5	3	0·17	4·25	$P>0.05$
Between "standard" and "unknown"	0·02	1	0·02		
Regression	4·1	1	4·1	100·25	$P<0.01$
Deviation from parallelism	0·001	1	0·001		
Error	0·23	6	0·04		
Total ..	7·101	15			

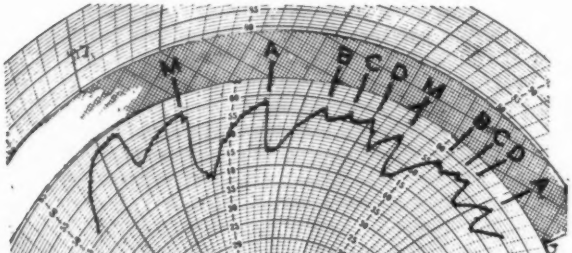

FIG. 7.—Part of Latin square assay of methanthelinium and atropine. The antagonists were injected together with a constant dose of methacholine (5 μg.). M=methacholine 5 μg.; A=atropine 1 μg.+M; B=atropine 3 μg.+M; C=methanthelinium 0·3 μg.+M; D=methanthelinium 0·1 μg.+M. Each large horizontal division corresponds to 0·5 *p*H units.

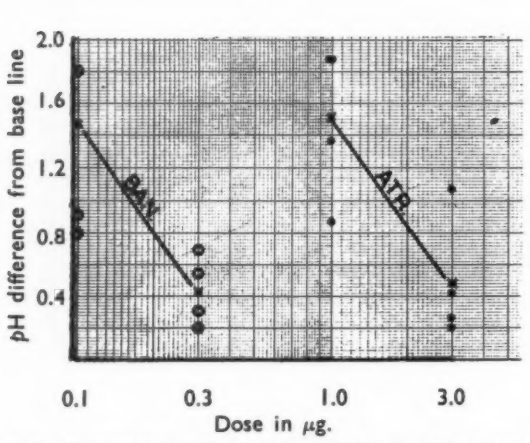

FIG. 8.—Regression lines fitted to assay of Fig. 7. ATR=Atropine; BAN=Methanthelinium.

TABLE V

ESTIMATED ACTIVITY, FIDUCIAL LIMITS AND λ VALUE OF THREE ATROPINE-LIKE DRUGS

Assay	Activity Ratio	Fiducial Limits (P=0·95)	λ
Methanthelinium/atropine (without recovery dose). 2 rats	10·7	8·2–14·1	0·09
Methanthelinium/atropine (with recovery dose). 4 rats	10·7	4·4–29·1	0·25
Atropine methylnitrate/atropine (without recovery dose). 2 rats	3·1	2·0–4·8	0·14

TABLE VI

ANALYSIS OF VARIANCE OF THE RESULTS OF THE ASSAY OF ATROPINE METHYLNITRATE AND ATROPINE

Source of Variation	Sum of Squares	d.f.	Mean Square	F	
Between groups	1·18	3	0·39	11·2	$P<0.01$
Dose order	0·28	3	0·09	2·7	$P>0.05$
Between "standard" and "unknown"	0·002	1	0·002		
Regression	1·69	1	1·69	48·2	$P<0.01$
Deviation from parallelism	0·09	1	0·09	2·6	$P>0.05$
Error	0·21	6	0·035		
Total ..	3·452	15			

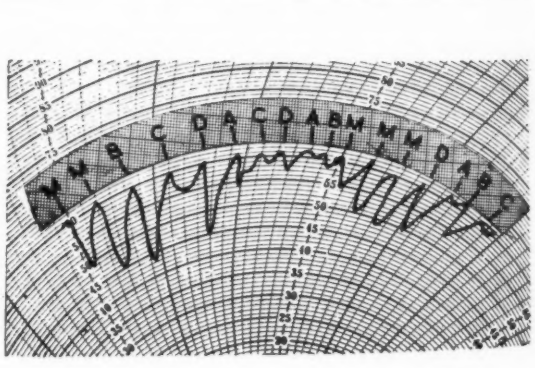

FIG. 9.—Part of assay of atropine and atropine methylnitrate. Three blocks of 4 doses injected in the same rat. M=methacholine 10 μg.; A=atropine methylnitrate 0·3 μg.+M; B=atropine 0·3 μg.+M; C=atropine 0·9 μg.+M; D=atropine methylnitrate 0·1 μg.+M.

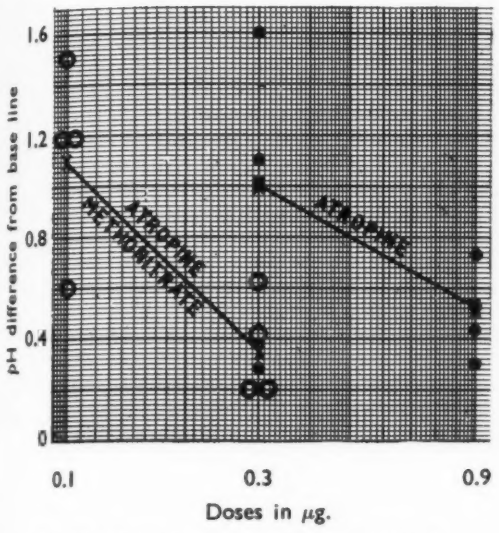

FIG. 10.—Regression lines fitted to assay of Fig. 9.

received one block and the other three blocks of four doses. The latter part of the assay is illustrated in Fig. 9. It shows a cumulative effect of successive doses of inhibitor which at first sight would seem to invalidate the result. Analysis of variance, however, shows that the result is nevertheless valid, since the F value for regression is highly significant and there is no significant deviation from parallelism (Fig. 10) (Table VI). The activity ratio of atropine methylnitrate and atropine was 3.1 with fiducial limits 2.0 and 4.8 at the 5% level.

DISCUSSION

The object of this work has been to devise statistically valid assays for inhibitors of gastric secretion making use of the continuous perfusion method described in an earlier paper (Ghosh and Schild, 1958). In this paper only inhibitors with a transient action are discussed. Assays of long-lasting inhibitors of the urogastrone type will be discussed elsewhere (Ghosh, Gregory, and Schild, unpublished observations).

Vasopressin is an example of a short-lasting inhibitor whose effect is probably due to vasoconstriction. A series of doses of vasopressin constituting one or more assay blocks can be given in the same preparation with relatively little interference from one dose to the next, although the more consistent results (lower λ values) obtained by interpolating doses of histamine suggest that interference between successive doses of inhibitor is not completely eliminated. But lower λ values have to be balanced against loss of time, and, in this sense, the method with the interpolated doses of histamine is less efficient.

Atropine-like drugs produce more persistent effects than vasopressin, but it is nevertheless possible to administer repeated doses in one preparation and perform a satisfactory assay. Estimates of relative activity and fiducial limits could be obtained by the use of only two rats for a complete assay. In spite of interaction between successive doses the dose/response relation of the inhibitor is not obliterated; in each experiment the larger dose produced a significantly greater effect than the smaller. A Latin square design was adopted in order to equalize dose order and thus correct to some extent for persistence of effects. This design seemed justified, since in the analysis of variance the mean square for dose order considerably exceeds error, although the F ratio does not reach the 5% level of significance.

In terms of number of animals required to achieve a given degree of accuracy the present assay method is much more efficient than the assay method in the pylorus-ligated rat. The efficiency of an assay can be expressed by n/λ^2 where n is the number of doses administered to one animal. In the present assays average values for n and λ are 8 and 0.16. No λ values have been published for the pylorus-ligation method, but a rough estimate on the basis of the published results of Visscher *et al.* (1954) gives a value of $\lambda = 0.85$ with $n = 1$. The ratio of efficiencies of the two methods thus works out at 190.

TABLE VII
ACTIVITY RATIOS FOR SECRETORY INHIBITORS

Inhibitor	Administration	Stimulant	Preparation	Activity Ratio	Reference
Methanthelinium/atropine ..	Subcutaneous	Histamine	Gastric secretion (dog)	1/20	Benjamin *et al.* (1950)
	Intravenous	Spontaneous secretion	Gastric secretion (pylorus-ligated rat)	1/10	Visscher *et al.* (1954)
	,,	Methacholine	Gastric secretion (rat stomach)	10	Present work
	,,	Carbachol	Salivary secretion (cat)	2·2	Johnson and Wood (1954)
Atropine methyl iodide/atropine	Intramuscular	Spontaneous secretion	Gastric secretion (pylorus-ligated rat)	10	Shea (1956)
Atropine methylnitrate/atropine	Intravenous	Methacholine	Gastric secretion (rat)	3·1	Present work

Such calculations have of course only limited validity, since different assay methods are not interchangeable and may give widely different results, especially when different stimulants are used. This is shown in Table VII, which gives the activity ratios for methanthelinium-atropine and methylatropine-atropine as secretory inhibitors when tested by different quantitative methods.

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STUDIES OF THE ACTION OF HYPOGLYCIN-A, AN HYPOGLYCAEMIC SUBSTANCE

BY

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Some biological effects of hypoglycin-A, a compound isolated from the fruit of *Blighia sapida*, have been investigated. Administration of this compound to animals caused drowsiness progressing to coma, and when large doses were given the animals died. For the rat, the oral and intraperitoneal LD50 values were 98 and 97 mg./kg. respectively. Fasting increased the toxicity considerably. The most outstanding biochemical change produced by hypoglycin-A was a delayed hypoglycaemia, the depth of which was related to the dose. The hypoglycaemia was preceded by exhaustion of liver glycogen. There were also smaller decreases in the glycogen stores of the heart, skeletal muscle and kidney, without any increase in blood pyruvate or lactate. Hypoglycin-A lessened the effect of adrenaline on blood glucose and decreased both glucose tolerance and insulin sensitivity. Hypoglycin-A also decreased the oxygen consumption and carbon dioxide production of the intact rat. All these effects are consistent with the hypothesis that the primary action of hypoglycin-A is the interference with glycogen production by the liver.

Hypoglycin-A is a compound which may be isolated from the fruit of *Blighia sapida*, a tree growing commonly in the West Indies, where it was introduced from West Africa in 1776 (Broughton, 1794). In Jamaica it is known as the "ackee." The fleshy part (aurillus) of the fruit of the ackee is eaten in Jamaica and West Africa, and at times may constitute an important item in the diet.

There is, nevertheless, a native tradition that the ackee may be poisonous. Bowrey (1892) attributed the cause of 5 fatal cases of poisoning to eating ackee. Since then, it has been suggested that the ingestion of this fruit may be a cause of an acute and often fatal disease known in Jamaica as the "vomiting sickness" (Scott, 1916; Hill, 1952). Jordan and Burrows (1937) and Evans and Arnold (1938) obtained extracts of ackee which caused vomiting and death in experimental animals. Although the relationship between vomiting sickness and the ingestion of ackee fruit was not accepted by all (Williams, 1952; Barnett, 1954), there was no doubt as to the presence of toxic substances in the ackee. Two such substances, hypoglycin-A and B, were isolated in 1954 (Hassall, Reyle, and Feng) and were shown to cause severe hypoglycaemia, depletion of liver glycogen and death. These findings agree well with those found in vomiting sickness (Hill, Bras, and Clearkin, 1955; Patrick, 1954).

Preliminary work suggested that hypoglycin-A and B were polypeptides (Hassall *et al.*, 1954; Hassall and Reyle, 1955). It now appears, however, that hypoglycin-A is probably an amino acid of novel structure (Hassall, personal communication) with the empirical formula of $C_7H_{11}NO_2$ (Holt, Leppla, Kroner and Holt, 1956). Earlier suggestions that the active principle of the ackee was a glycoside (Jordan and Burrows, 1937) or a saponin (Evans and Arnold, 1938) must be abandoned.

The present study was undertaken to provide a more complete account of the pharmacological and biochemical effects of hypoglycin-A on animals. Hypoglycin-A was studied because it is much more active biologically than hypoglycin-B and occurs in both the seeds and the edible aurillus of the ackee. Hypoglycin-B is less active and occurs in the seeds only.

MATERIAL AND METHODS

The hypoglycin-A used in the present study was isolated from ackee seed according to the method previously described by Hassall and Reyle (1955). The method involves the preparation of an aqueous extract of the seeds, precipitation of starchy material

by the addition of alcohol, and fractionation of the material in the supernatant fluid by means of ion exchange resins. The fractionation process was guided by toxicity tests and by the hypoglycaemic action of the fractions. The purity of the final product was checked by paper chromatography. In all cases there were ninhydrin-positive contaminants estimated to constitute not more than 2% of the total sample.

White rats were used for all *in vivo* experiments, except where otherwise stated. Hypoglycin-A was administered to the animals in the form of freshly-prepared aqueous solutions containing 10 to 30 mg. of hypoglycin-A/ml. Studies of the blood chemistry of rats were conducted with blood obtained from the tail. Blood glucose was determined by the colorimetric method of Nelson (1944). Blood lactate was determined by a micro-modification of the method of Barker and Summerson (1941) and pyruvate was determined by a micro-modification of the method of Friedemann and Haugen (1943). Glycogen was determined by the technique of Good, Kramer and Somogyi (1933). The assay for glucose-6-phosphatase was carried out by the procedure of Langdon and Weakley (1955) and the assay for phosphorylase by the method of Sutherland (1955). Plasma-urea, acid-soluble phosphate, chloride and cholesterol levels were determined by the procedures outlined by King (1946) with minor modifications. Estimation of the glucose uptake by the isolated rat diaphragm was carried out using the method described by Randle (1956) with minor modifications.

Results

Toxic Effects.—Observations on the toxic effects of lethal doses of hypoglycin-A (100 to 500 mg./kg., intraperitoneally) were made in kittens, guinea-pigs and white rats. Drowsiness, lachrymation and secretion from the nose and mouth occurred within 30 min. to 1 hr. after administration. Vomiting, starting 30 min. to 1 hr. after administration and lasting 2 to 4 hr., was observed frequently in kittens. The drowsiness progressed with time and the animals eventually became comatose. At this time, respiration was slow and cyanosis was usually evident at the terminal stage. Convulsions were not seen. Time of death varied with the dose administered.

Autopsies performed immediately after death revealed few gross pathological changes. The visceral veins and the right side of the heart were distended and filled with dark-coloured blood. The left ventricle was usually contracted. There was slight congestion in the liver, lungs, spleen, and kidneys, but otherwise these organs appeared normal. Histological sections of the liver, spleen, lungs, heart, kidneys, and suprarenal glands stained with haematoxylin and eosin revealed no significant pathological changes. Liver sections stained with Best's carmine showed a marked reduction in glycogen granules. Sections of the pancreas stained with Gomori's chrome-alum haematoxylin and phloxine, and Gomori's aldehyde fuchsin and phloxine, revealed a reduction of the granules of the alpha cells of the islets. This result has been described elsewhere (Feng, 1957).

Effects on Respiration and Blood Pressure.—Respiration and carotid arterial pressure were recorded from 5 kittens (400 to 800 g.) anaesthetized with pentobarbitone. Repeated intravenous injections (20 to 150 mg./kg.) of hypoglycin-A caused no immediate changes in the rate and depth of respiration or on the blood pressure. Just before death, which occurred after 4 to 5 hr., there was a slowing of the rate of respiration and a sudden drop in blood pressure.

Determination of Acute Toxicity.—The toxicity of hypoglycin-A on fed rats of either sex was determined by administering graded doses of the compound orally or intraperitoneally to groups of six rats (body weight 100 to 200 g.) and noting the mortality 24 hr. later. The results are shown in Table I. The LD50 values of hypoglycin-A administered orally and intraperitoneally were calculated by the probit transformation described by Burn, Finney, and Goodwin (1950), and were found to be almost identical, being 98 and 97 mg./kg. respectively with fiducial limits of 85 to 111 mg./kg. body weight.

Table I

ACUTE TOXICITY OF HYPOGLYCIN-A TO RATS

Mortality was observed for 48 hr.

Dose (mg./kg.)	Mortality	
	Oral	Intraperitoneal
50	0/6	0/6
60	0/6	1/6
70	1/6	1/6
80	2/6	1/6
90	3/6	2/6
100	3/6	2/6
110	3/6	4/6
120	5/6	5/6
130	4/6	5/6
140	5/6	6/6
150	6/6	6/6

A second series of experiments was made to compare the mortalities of groups of rats which had either been fed or fasted for 48 hr. before giving hypoglycin-A intraperitoneally. The results (Table II) did not permit an accurate estimation of the LD50, but it is evident that fasting rendered the rats much more susceptible to the toxic action of hypoglycin-A.

TABLE II

INTRAPERITONEAL TOXICITY OF HYPOGLYCIN-A TO FED AND FASTED RATS

Food was withheld from the fasted rats for 48 hr. before injection. Mortality was observed for 48 hr.

Dose (mg./kg.)	Mortality	
	Fed Rats	Fasted Rats
30	—	0/6
50	0/6	6/6
70	2/6	6/6
90	3/6	—
110	2/6	—
130	6/6	—

Effects on Blood Glucose, Lactate, and Pyruvate.—Blood glucose estimations following the administration of hypoglycin-A to rats, rabbits, and guinea-pigs showed that a lethal dose was followed by profound hypoglycaemia with values for blood glucose below 30 mg./100 ml. just before the death of the animal. Fig. 1 shows the pattern of blood sugar response after the administration of graded oral doses of hypoglycin-A to rats. There was occasionally an initial slight hyperglycaemia of 10 to 30 mg./100 ml. above normal, followed by a marked hypoglycaemia to values as low as 10 mg./100 ml. The time of onset and degree of hypoglycaemia were related to the dose. It was also noted that the progress of the drowsiness and coma and the time of death were related to the degree of hypoglycaemia. The pattern of the changes in blood sugar was the same whether the hypoglycin-A was administered orally or intraperitoneally.

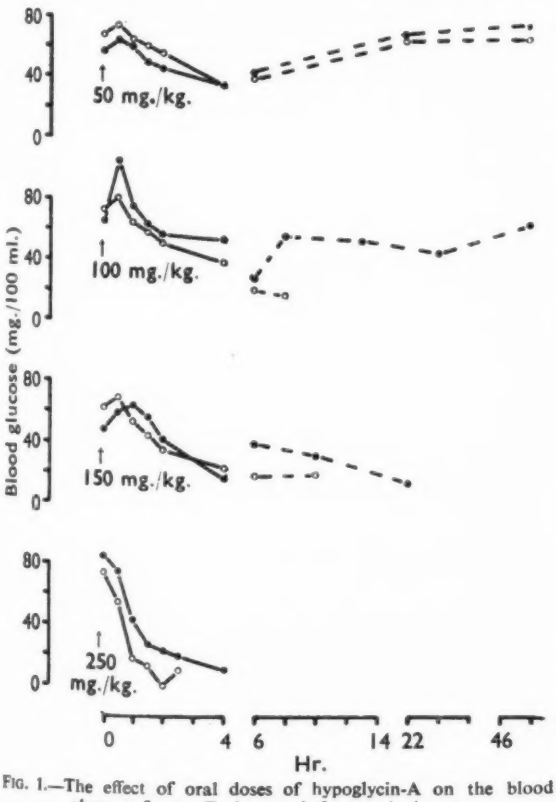

FIG. 1.—The effect of oral doses of hypoglycin-A on the blood glucose of rats. Each curve is from a single rat.

Blood lactate and pyruvate measurements were carried out on rats to which lethal doses (250 mg./kg.) had been administered by intramuscular injection. No outstanding changes were noted in the concentrations of these metabolites within the first 6 hr. after the administration of the hypoglycin-A although the blood sugar had fallen to 30 mg./100 ml. in this time.

Effects on Glycogen Stores, Liver Glucose-6-phosphatase and Phosphorylase.—A study was made of the glycogen concentrations of liver, heart, skeletal muscle and kidney in fed and fasted control rats and in fed and fasted rats to which a lethal dose (250 mg./kg.) of hypoglycin-A had been administered. Table III shows average values for the 4 groups of rats. The dose was administered intramuscularly 6 hr. before the glycogen determination. It is apparent that hypoglycin-A caused a decrease in the glycogen stores of some tissues whether the rats had been fed or fasted. The most striking effects were on the liver and the heart, and there was a small decrease in skeletal muscle glycogen. It is noteworthy that fasting in itself caused an increase in heart glycogen, whereas hypoglycin-A administration had the opposite effect.

TABLE III

THE EFFECT OF HYPOGLYCIN-A ON THE GLYCOGEN CONTENT OF RAT TISSUES

Food was withdrawn from the fasted animals 48 hr. before the intramuscular injection of hypoglycin-A (250 mg./kg.). Glycogen was determined in the tissues 6 hr. after the dose. Each numeral is the average for 4 rats.

Group of Rats	Glycogen (g./100 g. Tissue, Wet Weight)			
	Liver	Skeletal Muscle	Heart	Kidney
Fed; control	3·88	0·49	0·35	0·07
Fed; hypoglycin-A ..	0·07	0·32	0·08	0·06
Fasted, control	0·24	0·32	0·60	0·12
Fasted; hypoglycin-A ..	0·07	0·23	0·07	0·07

An increase in the activities of glucose-6-phosphatase or phosphorylase could conceivably explain the reduction in liver glycogen which occurs as a result of administration of the drug. For this reason, liver homogenates were prepared from rats which had been given lethal doses, and the activities of these enzymes in the homogenates were assayed. No evidence was obtained for

significant changes in the activities of these enzymes during the first 6 hr. after administration of hypoglycin-A, whereas the liver glycogen content decreased progressively during that period.

Effects on Glucose Tolerance.—Previous results (Patrick, 1954) had indicated that there was probably a decrease in glucose tolerance. This finding has been confirmed and extended in the present study. Glucose (3 g./kg.) in aqueous solution was administered intraperitoneally to 10 rats, and the changes in blood glucose were determined. On the following day, the same rats were given first a sublethal dose of hypoglycin-A (70 mg./kg.), then 1 hr. later the glucose administration and blood sugar determinations were repeated. A typical result is shown in Fig. 2. The decreased glucose tolerance illustrated resembled that found in diabetes, which would indicate a defective uptake of glucose from the blood by tissues.

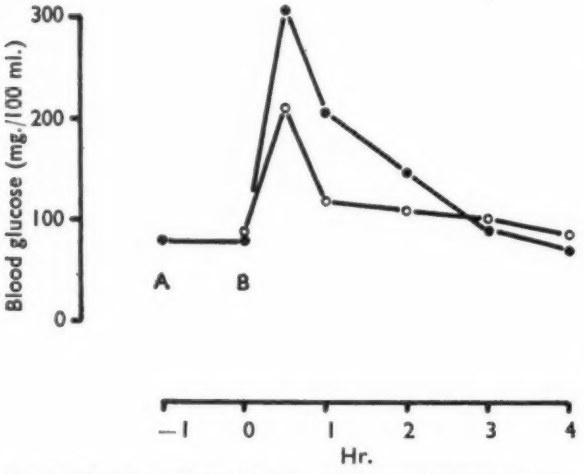

FIG. 2.—The effect of hypoglycin-A on the glucose tolerance of a rat. O, Blood glucose curve of rat after a dose of 3·0 g./kg. (i.p.) of glucose at B; ●, curve from the same rat when 70 mg./kg. (i.p.) of hypoglycin-A was given (at A) 1 hr. before the glucose (at B).

Effects on the Response of Blood Sugar to Adrenaline and Insulin.—Tests of the effects of adrenaline and insulin on blood glucose were carried out on groups of 6 rats. A predetermined dose of adrenaline (0.2 mg./kg.) or insulin (3 units/kg.) was administered to the rat and the changes in blood glucose were followed. On the following day the hormone administration and blood sugar determination were repeated using the same rats 1 hr. after they had been given a sublethal dose of hypoglycin-A. The drug was administered intraperitoneally in doses of 70 mg./kg. for the adrenaline test and 30 mg./kg. for the insulin test. There was a decrease in the effect of adrenaline on blood sugar after administration of hypoglycin-A (Fig. 3), and this may probably be ascribed to the decrease in liver glycogen. The drug also caused a slight but persistent decrease in insulin sensitivity (Fig. 4). It would thus appear that at this dose the effects of insulin and hypoglycin-A are antagonistic.

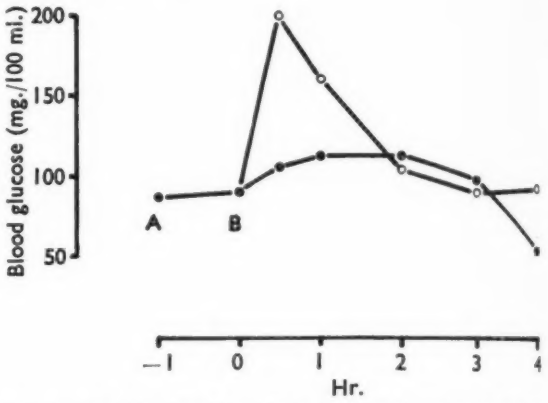

FIG. 3.—The influence of hypoglycin-A on the hyperglycaemic effect of adrenaline on a rat. O, Control blood glucosel response to adrenaline; ● response to adrenaline in the same rat after the administration of hypoglycin-A. At A, hypoglycin-A 70 mg./kg. (i.p.). At B, adrenaline 0·2 mg./kg. (i.p.).

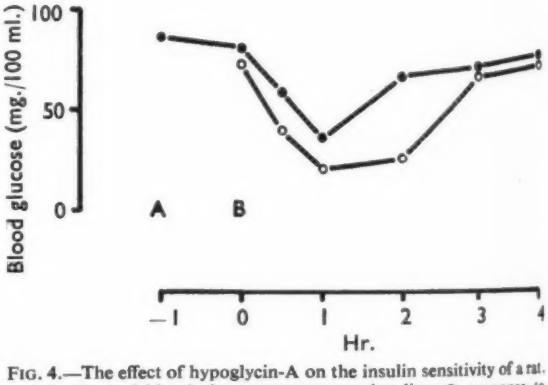

FIG. 4.—The effect of hypoglycin-A on the insulin sensitivity of a rat. O, Control blood glucose response to insulin; ●, response to insulin in the same rat after the administration of hypoglycin-A. At A, hypoglycin-A 30 mg./kg. (i.p.). At B, insulin 3 i.u./kg. (i.p.).

Effects on Oxygen Consumption and Carbon Dioxide Production of Rats.—Experiments were carried out on the oxygen consumption and carbon dioxide production of normal rats, rats which had been given hypoglycin-A and rats which had been given insulin. After administration of the drug, the rats were placed in a metabolism chamber of the type described by Robbie (1948). Pressure readings for the calculation of the oxygen consumption and carbon dioxide production were obtained hourly for 6 hr.

Figs. 5 and 6 illustrate the average results for groups of 4 rats subjected to each treatment. The dose of hypoglycin-A was 150 mg./kg., and the dose of insulin was 8 units/kg., both given intramuscularly. The actions of insulin and hypoglycin-A on metabolism were completely different. Insulin caused marked increases in both oxygen consumption (Fig. 5) and carbon dioxide (Fig. 6) production during the hypoglycaemic stage, when the respiratory quotient rose to 1.0; hypoglycin-A caused decreases in both oxygen consumption and carbon dioxide production during the stage of its hypoglycaemic action.

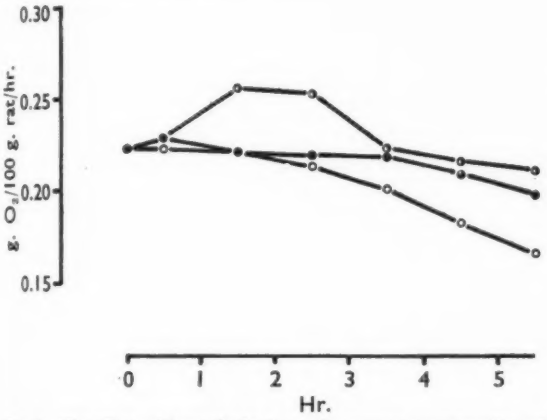

FIG. 5.—The effect of hypoglycin-A on the oxygen consumption of rats. ●, Control; ◑, after insulin 8 i.u./kg.; O, after hypoglycin-A 150 mg./kg. Average results of 4 animals.

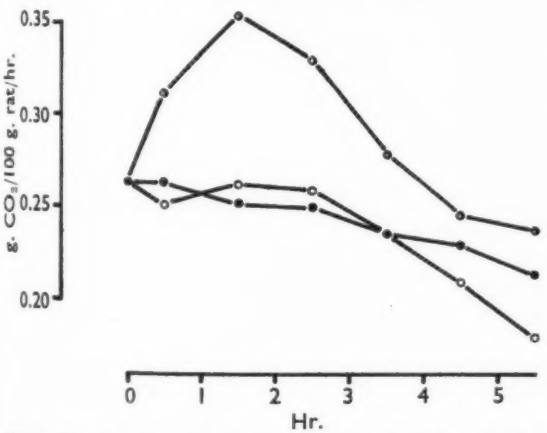

FIG. 6.—The effect of hypoglycin-A on the carbon dioxide production of rats. ●, Control; ◑, after 8 i.u./kg. of insulin; O, after 150 mg./kg. of hypoglycin-A. Average results of 4 animals.

Effect on Other Blood Constituents.—Repeated determinations of plasma urea, acid-soluble phosphate, chloride, and cholesterol levels were made on groups of 8 rats after the administration of a lethal dose of hypoglycin-A (150 to 300 mg./kg.). No significant changes were observed except some increase in plasma urea and acid-soluble phosphate just before the death of the animals.

Lack of in vitro *Effects.*—Attempts to demonstrate *in vitro* effects of hypoglycin-A on isolated tissues have not succeeded. Concentrations of 1:1,000 to 1:1,000,000 of the drug had no effect on the spontaneous contraction of intestine or uterus of the rat or guinea-pig. Also, hypoglycin-A in these concentrations did not affect the characteristic actions of adrenaline, acetylcholine, histamine or pituitary (posterior lobe) extract on smooth muscle.

The uptake of glucose by the isolated rat diaphragm appeared to be unaffected by the presence in the medium of 2 mg./ml. of hypoglycin-A.

The metabolism of isolated liver slices also did not appear to be affected by the presence of hypoglycin-A in the medium at a concentration of 1 to 2 mg./ml. The QO_2 and RQ of the liver slices, and their ability to synthesize glycogen, were unchanged.

Discussion

The finding of hypoglycaemia and low liver glycogen concentrations in animals after hypoglycin-A administration and in patients suffering from "vomiting sickness" is evidence in support of the theory that the ingestion of the ackee is a cause of the disease in man. Two considerations, however, make it evident that this theory must be qualified before it can be accepted. Firstly, the fruit of the ackee is commonly eaten by Jamaicans, yet the incidence of vomiting sickness is very low. Secondly, it would appear likely from the toxicity data in this paper that a very large amount of ackee fruit would have to be ingested by normal people before toxic symptoms would appear. The demonstration that the toxicity of hypoglycin-A is much increased by fasting rats, or by feeding them a deficient diet (Feng and Kean, 1955), may be significant in view of the fact that the "vomiting sickness" occurs frequently in children with a history of malnutrition (Jelliffe and Stuart, 1954).

Several inferences regarding the metabolism of hypoglycin-A may be drawn from the present work. The fact that the effects of hypoglycin-A are similar, whether the compound is administered orally or intraperitoneally, suggests that no destruction of the drug occurs in the gastro-intestinal tract and absorption is efficient. The failure to demonstrate effects of hypoglycin-A on isolated tissues *in vitro* leads one to speculate

whether the substance must first be metabolized or activated in the body before it exerts its characteristic effects *in vivo*.

Present evidence indicates that the pharmacological action of hypoglycin-A is confined to its effects on carbohydrate metabolism and the death of the animals may be ascribed to hypoglycaemia. Unlike insulin, the hypoglycaemic action of hypoglycin-A is delayed, and its effects on muscle glycogen, oxygen consumption, carbon dioxide production and glucose tolerance are all the reverse of insulin effects. Furthermore, hypoglycin-A in small doses actually antagonizes the usual response of blood sugar to insulin.

There is no evidence to indicate that hypoglycin-A causes increased utilization of carbohydrate; there was no increase of blood pyruvate or lactate, muscle glycogen or respiratory carbon dioxide. It must be concluded that hypoglycin-A interferes with carbohydrate production by the liver. This agrees with the previous suggestion (Patrick, 1954) that hypoglycin-A hypoglycaemia is a result of the exhaustion of liver glycogen.

The authors wish to express their gratitude to members of the Department of Pathology of the University College of the West Indies for some histological examinations, and to the Department of Chemistry for the suppy of hypoglycin-A. This study was supported by the Government of Jamaica from a grant for the "Vomiting Sickness Study."

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A COMPARISON OF SOME ACTIVITIES OF ARGININE VASOPRESSIN AND LYSINE VASOPRESSIN ON KIDNEY FUNCTION IN CONSCIOUS DOGS

BY

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Comparison was made on conscious trained dogs of the effect of highly purified arginine vasopressin and lysine vasopressin on the rate of urine flow, the excretion rate of Na and K and the renal clearances of creatinine and diodone, and of the actions of the vasopressins when administered together with oxytocin. The vasopressins were used in doses causing a maximal antidiuresis.

The results showed, first, that the antidiuretic potency of arginine vasopressin was greater than that of lysine vasopressin and that its action lasted longer; second, that during water diuresis arginine vasopressin increased only Na excretion while lysine vasopressin increased both Na and K excretion; third, that arginine vasopressin had a potency about 5 to 6 times that of lysine vasopressin in antagonizing the augmentor effect of oxytocin on glomerular filtration rate and renal plasma flow.

It has been shown that at least two vasopressins can be found in mammals, arginine vasopressin from beef and lysine vasopressin from hog (Turner, Pierce, and du Vigneaud, 1951; Poponoe, Lawler, and du Vigneaud, 1952). Van Dyke, Engel, and Adamsons (1956) compared a number of the activities of these two vasopressins and found that the ratio of pressor potency to antidiuretic potency is 1.0 for arginine vasopressin and 6 for lysine vasopressin when the antidiuretic potency is determined by intravenous injection in hydrated unanaesthetized dogs. On the other hand, as compared with the USP standard, lysine vasopressin and arginine vasopressin on intravenous injection were equally potent as pressor agents in anaesthetized rats and dogs. This difference in the potency ratio of the two substances suggested the possibility that the activities considered were related to different parts of the molecular configuration. It was not known whether lysine vasopressin had certain of the other activities of arginine vasopressin, and it seemed of interest to make a further comparison of the biological properties and potency ratios of the two vasopressins in the hope that ultimately some clue might be obtained as to the chemical nature of the processes involved in their actions. A comparison was made of the power of the two vasopressins to affect the excretion rate of Na and K during diuresis and at low rates of urine flow, and of their relative capacity to antagonize the augmentor effect of oxytocin on glomerular filtration rate and renal plasma flow. The first of these activities was investigated because Anslow and Wesson (1955) and Brooks and Pickford (1957) found that in dogs arginine vasopressin increased electrolyte excretion during water diuresis, but was ineffective at low rates of urine flow. The second comparison was made since Brooks and Pickford (1957) found that arginine vasopressin was highly effective at antagonizing the increased renal plasma flow induced by the administration of oxytocin.

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METHODS

A total of rather more than 90 observations were made. Most of the observations were made on two trained conscious bitches, Theone (21 kg.) and Sophie (18 kg.), but a few were also made on 3 other dogs. They were given 15 to 20 ml. tap water/kg. body weight 2 to 2½ hr. before each experiment, and observations were made at about the same time in the afternoon. Diuresis was induced by a second dose of 20 ml./kg. warm tap water given by stomach tube. A number of observations were made at a "resting

rate" of urine flow when the dogs were hydrated as usual but were not given the second dose of water at the time observations were begun. The urine was collected through a self-retaining catheter into dry graduated tubes which had been carefully washed in Na and K free water. In both dogs the dorsal perineum was slit to facilitate catheterization. The urine collection periods were of 10 to 20 min. duration, usually 10 min. during diuresis and 20 min. during resting rates of flow. The dead space of the urine collecting system was reduced as much as possible by keeping the external part of the catheter short. Intravenous injections were made into the saphenous vein and infusions into the cubital vein. When blood samples were needed these were obtained from the saphenous vein.

For the clearance experiments diodone (0.4 to 0.6 ml. of a 35% w/v solution/kg. body weight) and creatinine (0.2 to 0.3 g./kg.) were given subcutaneously 30 to 40 min. before the induction of diuresis. This subcutaneous administration was found to maintain for a period an adequate and fairly steady plasma concentration of both substances; consequently in calculating the clearances little adjustment was necessary to allow for dead space (Chinard, 1955).

Vasopressins and oxytocin were given either as single intravenous injections or as intravenous infusions. The single injections were of 1 ml. 0.9% NaCl solution containing the required amount of hormone(s). Infusions were given at a constant rate of 3 ml./hr. Highly purified natural vasopressins were used throughout. These were available owing to the kindness of Dr. du Vigneaud and Dr. van Dyke, and were standardized in pressor units. The oxytocin used was synthetic and this has been shown to be identical in its activities with natural oxytocin. On a few occasions highly purified natural oxytocin was used.

Diodone iodine of plasma and urine was estimated by the method of Alpert (1941). Creatinine in plasma and urine was estimated as suggested by Rehberg (1926) and by the colorimetric method of Folin (1914) respectively. Sodium and potassium were estimated by flame photometer.

Results

Antidiuresis and Electrolyte Excretion Following Injection of the Vasopressins

Preliminary tests on the two dogs chiefly used showed that, despite their different body weights, a single intravenous injection of 2 mU. arginine vasopressin produced a maximal antidiuresis, and that this was matched in degree by the administration of 7 mU. lysine vasopressin (Fig. 1), but recovery from antidiuresis caused by lysine vasopressin was more rapid than that from arginine vasopressin. Further comparison of the actions of the two hormones was based on the use of these equi-antidiuretic doses.

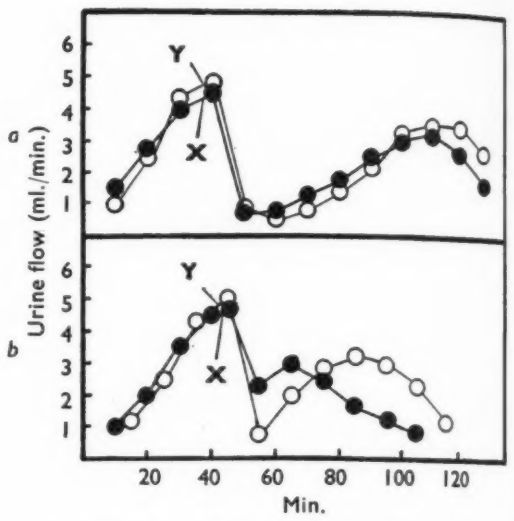

FIG. 1.—Antidiuretic activities of arginine and lysine vasopressins. *a*, (Theone), 400 ml. water by mouth at zero time. Arginine vasopressin injected intravenously, 2 mU. at *X* (●); 3 mU. at *Y* (○). *b*, Curve ● (Sophie), 350 ml. water by mouth at zero time; 5 mU. lysine vasopressin intravenously at *X*. Curve ○ (Theone), 400 ml. water by mouth at zero time; 7 mU. lysine vasopressin injected intravenously at *Y*.

Before considering the effect of the hormones on electrolyte excretion it is as well to make it clear that no significance was attached to a rise in excretion rate in a single immediately post-injection sample. A rise in two consecutive samples was possibly significant. No rise in electrolyte excretion ever followed control injections. When it is stated that the injection of hormone caused an increase in electrolyte loss this implies that the increased loss always occurred. Fig. 2 shows the rate of Na and K excretion during control observations when 1 ml. 0.9% NaCl solution was injected intravenously. This may be compared with the typical effect on Na and K excretion of arginine vasopressin and lysine vasopressin injected during diuresis (Fig. 3). Both vasopressins caused a considerable, though short-lived, rise in Na excretion. The effect of 2 mU. arginine vasopressin on K loss was of no significance on this occasion, though it was found that 3 mU. arginine vasopressin moderately increased the excretion rate of K in the two 10 min. samples following on those injections. However, in terms of antidiuresis 3 mU. arginine vasopressin was a supramaximal dose and therefore not comparable with the effect of 7 mU. lysine vasopressin which regularly raised the excretion rate of K, sometimes for as long as 40 to 50 min. Lysine vasopressin infused during diuresis at a rate of 0.01 to 0.02 mU./kg./min. also caused an increase in the

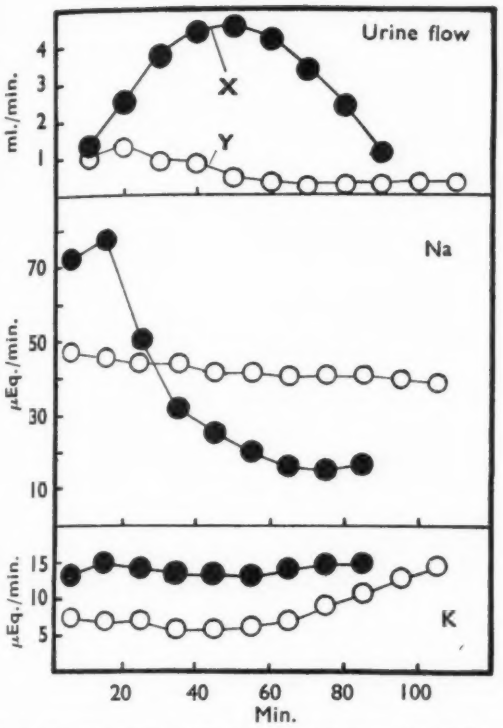

FIG. 2.—Excretion of Na and K during control observations (Theone). Curves ●, 400 ml. water by mouth at zero time: 1.0 ml. 0.9% NaCl solution injected intravenously at *X*. Curves ○, no water at zero time, 1.0 ml. 0.9% NaCl solution intravenously at *Y*.

excretion of both Na and K, the increase being maintained for the period of infusion.

At a resting rate of urine flow (when the dogs had been hydrated but were not diuretic at the time of observation) it was found that neither single injections nor infusions of arginine vasopressin appreciably nor consistently increased Na or K loss (Fig. 4). This is in agreement with the findings of Anslow and Wesson (1955) and Brooks and Pickford (1957). At the low rate of urine flow lysine vasopressin similarly had little action on the excretion rate of Na, but it caused an augmented loss of K both after single injections (Fig. 4) and more clearly during infusions.

Antidiuresis and Electrolyte Excretion Following Injection of the Vasopressins Together with Oxytocin

It will be shown later that both vasopressins antagonized the action of oxytocin on the renal blood vessels. For this reason it was thought as well to test if they also antagonized the effect of oxytocin on electrolyte excretion. Observations were made on the excretory response, at both high and low rates of urine flow, to the injection of oxytocin simultaneously with either arginine or

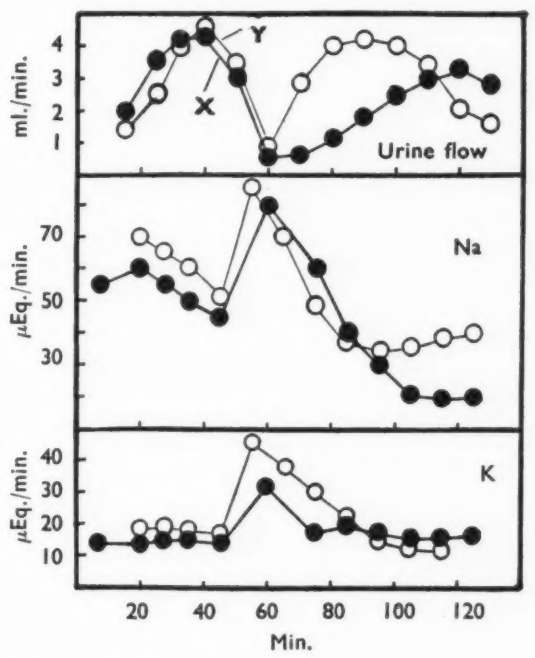

FIG. 3.—The effect of arginine vasopressin and lysine vasopressin on urine flow and on the excretion of Na and K during water diuresis. (Theone), 400 ml. water by mouth at zero time. Intravenous injection of 2 mU. arginine vasopressin at *X* (●); 7 mU. lysine vasopressin at *Y* (○).

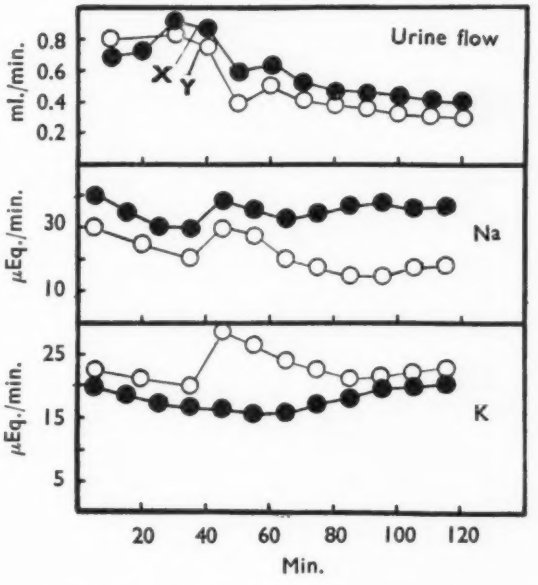

FIG. 4.—The effect of arginine and lysine vasopressins on urine flow and on Na and K excretion at a low rate of urine flow. (Theone), no water at zero time. Intravenous injection of 2 mU. arginine vasopressin at *X* (●); 7 mU. lysine vasopressin at *Y* (○).

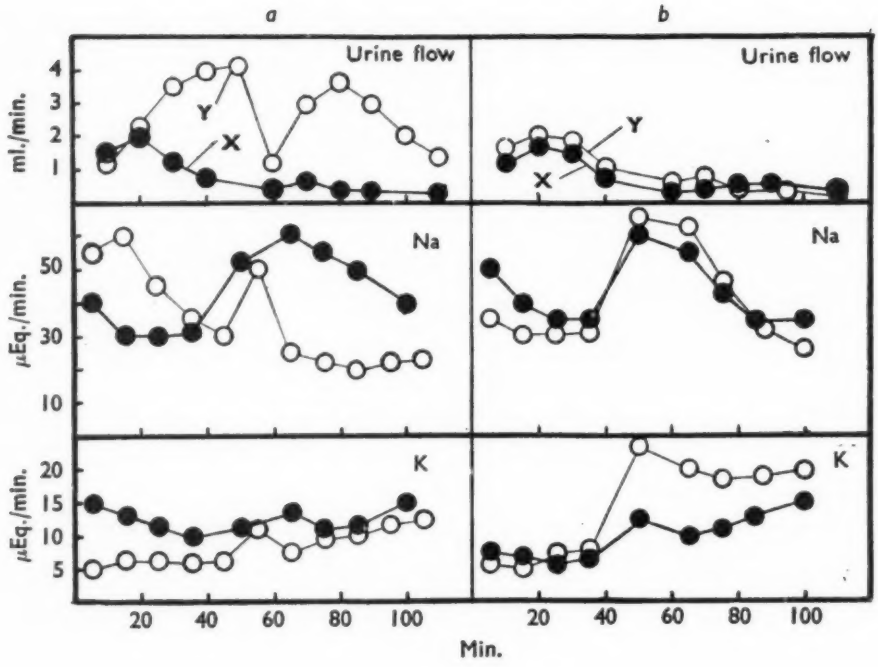

FIG. 5.—(*a*) The effect of oxytocin on urine flow and on Na and K excretion. Curves ○, water diuresis; curves ●, "resting" rate of urine flow. On both occasions 150 mU. oxytocin injected intravenously at times marked *X* and *Y*. (*b*) The effect of oxytocin plus arginine vasopressin and of oxytocin plus lysine vasopressin on urine flow and on Na and K excretion during "resting" rate of urine flow. Intravenous injections: 150 mU. oxytocin plus 2 mU. arginine vasopressin at *X* (●); 150 mU. oxytocin plus 7 mU. lysine vasopressin (○).

lysine vasopressin. The doses of oxytocin given were the same as those used below for testing the activity of the two vasopressins on the renal blood vessels. Confirming the results of Brooks and Pickford (1957) it was found that oxytocin, given either by infusion or as a single intravenous injection, raised the excretion rate of Na at a resting rate of urine flow, but was ineffective during diuresis (Fig. 5*a*). Following the administration, at a resting rate of urine flow, of oxytocin together with arginine vasopressin, the period of increased Na loss was shorter than that following oxytocin alone, but K excretion was not consistently changed (Fig. 5*b*). Lysine vasopressin, like arginine vasopressin, appeared to shorten the period of increased Na loss which followed oxytocin alone (Fig. 5*b*); on the other hand, lysine vasopressin and oxytocin given together always clearly enhanced the rate of K excretion (Fig. 5*b*) as compared with the action of either substance given alone (cf. Fig. 5*a* and Fig. 3). During water diuresis the administration of oxytocin together with arginine vasopressin shortened the duration of the expected antidiuresis and resulted in a sharper increase in loss of Na than followed arginine vasopressin alone (cf. Figs. 3 and 6). K excre-

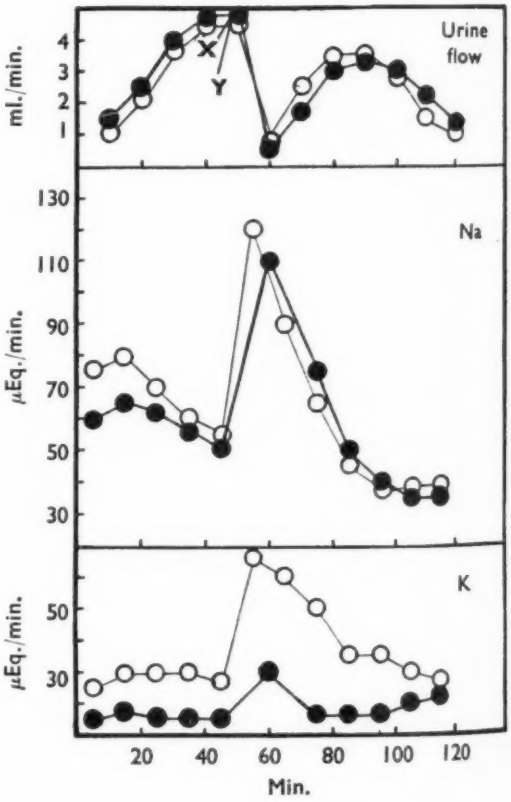

FIG. 6

FIG. 6.—The effect of oxytocin plus arginine vasopressin and of oxytocin plus lysine vasopressin on urine flow and on Na and K excretion during water diuresis. Intravenous injections; 150 mU. oxytocin plus 2 mU. arginine vasopressin at *X* (●); 150 mU. oxytocin plus 7 mU. lysine vasopressin at *Y* (○).

tion was not significantly altered by the simultaneous injection of arginine vasopressin and oxytocin (Fig. 6). When oxytocin was injected together with lysine vasopressin, again Na and possibly also K loss was enhanced as compared with the action of lysine vasopressin alone.

Effect of the Vasopressins Alone and Together with Oxytocin on Glomerular Filtration Rate and Renal Plasma Flow

It is generally agreed that Pitressin given in a dose just sufficient to cause maximal inhibition of water diuresis does not consistently alter either the glomerular filtration rate or renal plasma flow (Maxwell and Breed, 1951 ; Sellwood and Verney, 1955). Brooks and Pickford (unpublished observation) found that highly purified arginine vasopressin behaved in this respect like Pitressin. Nevertheless, these small doses of vasopressin abolish the increase of renal plasma flow caused by oxytocin (Brooks and Pickford, 1957), and it will be shown below that lysine vasopressin has to some extent the same property. Since the object was to compare the renal vascular actions of the two vasopressins, oxytocin was used in almost certainly unphysiological doses in order to ensure a large rise in renal plasma flow, and thus test to the full the power of the two vasopressins to antagonize this rise.

Control measurements during water diuresis in Theone showed that the clearance rates of creatinine and diodone increased by approximately 10 and 15% respectively with the onset of diuresis and declined as diuresis subsided. These clearance changes are of the same order as those found by Sellwood and Verney (1955). Brooks and Pickford (1957) using oxytocin in intravenous doses of 5 mU./kg. found that it caused an increase in renal plasma flow. In the present work doses of 7 to 10 mU./kg. were given and it was found that both glomerular filtration rate and renal plasma flow increased ; in Theone the former rose by 50 to 80% with a return to the initial value in 30 to 40 min., and the latter by 25 to 50% for a short time only. Fig. 7*a* records the results of one such

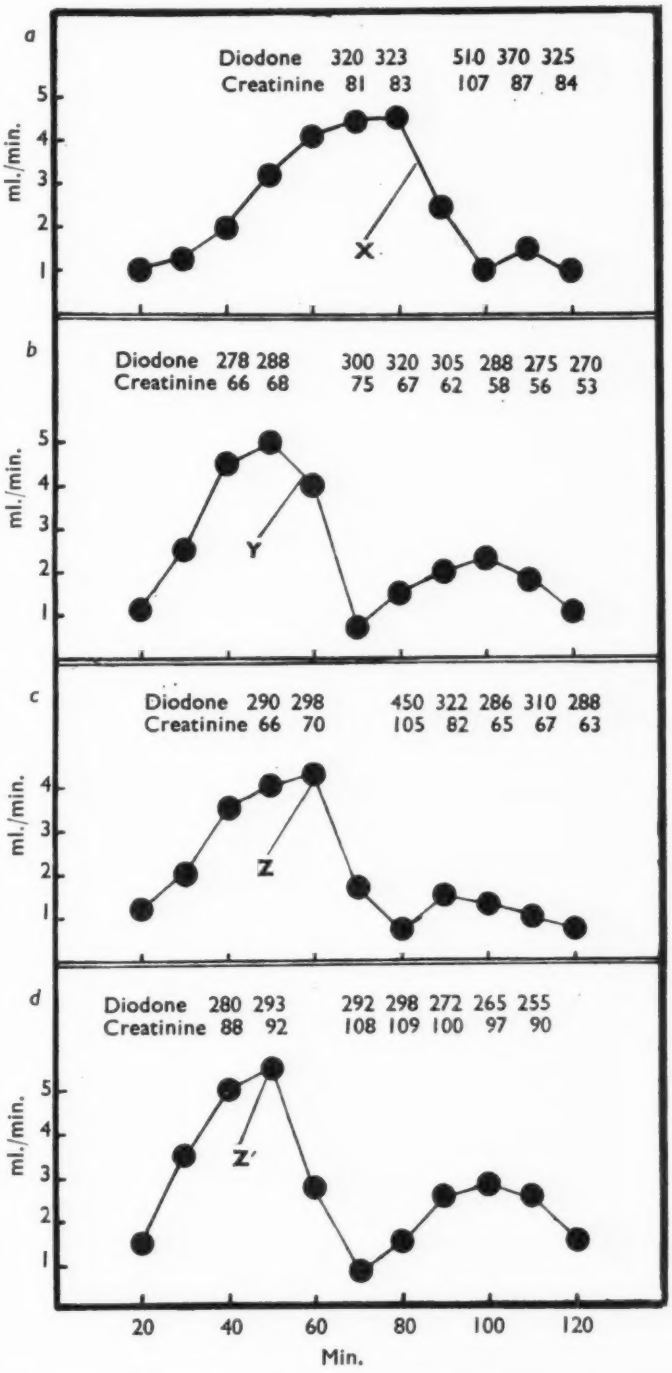

FIG. 7.—The effect of oxytocin and of oxytocin plus arginine vasopressin and oxytocin plus lysine vasopressin on the clearance rates of diodone and creatinine during water diuresis. All drugs were given by intravenous injections. **a**, 150 mU. oxytocin at ***X***; ***b*** 150 mU. oxytocin plus 2 mU. arginine vasopressin at ***Y***; ***c***, 150 mU. oxytocin plus 7 mU. lysine vasopressin at ***Z***; ***d***, 150 mU. oxytocin plus 10.5 mU. lysine vasopressin at ***Z'***. The related clearance values of diodone and creatinine are indicated above each curve in ml./min. Ordinates, urine flow in ml./min.; abscissae, time in min.

observation. This is similar to the findings of Dicker and Heller (1946) on rats. When 2 mU. arginine vasopressin was injected together with 150 mU. oxytocin the increase in clearance values was far less than when oxytocin was given alone (Fig. 7*b*). On the other hand, 7 mU. lysine vasopressin, that is, the antidiuretic dose-equivalent of 2 mU. arginine vasopressin, only slightly antagonized the action of oxytocin (Fig. 7*c*) and it was necessary to use 10.5 mU. lysine vasopressin to prevent the effect of oxytocin on renal plasma flow. Even this dose did not fully counteract the increase in glomerular filtration rate (Fig. 7*d*). On two occasions 7 mU. lysine vasopressin was injected 3 to 4 min. before the 150 mU. oxytocin; but this procedure did not increase the power of lysine vasopressin to antagonize the action of oxytocin.

Discussion

Under the conditions of the present observations 2 mU. arginine vasopressin and 7 mU. lysine vasopressin caused a maximal antidiuresis, but that due to lysine vasopressin lasted for a shorter time. The 1/3.5 ratio of antidiuretic potency of arginine/lysine vasopressin is lower than that (1/6) found by van Dyke, Engel, and Adamsons (1956), but this may perhaps be due to certain differences in procedure. Both sets of results show that in the conscious dog lysine vasopressin is a weaker antidiuretic than arginine vasopressin. As far as the electrolyte excreting properties of the vasopressins are concerned, when given in the maximal antidiuretic dose both increased Na excretion to the same extent during water diuresis, but lysine vasopressin also raised the excretion of K. Arginine vasopressin only increased K loss in addition to that of Na if given in supramaximal antidiuretic dose. The same results were seen following infusions, namely that lysine vasopressin was the more effective at increasing K loss.

In the present work oxytocin was used in fairly large doses, namely 7 to 10 mU./kg. According to an assay made in dogs by Abrahams and Pickford (1954), an osmotic stimulus which caused maximal antidiuresis brought about the liberation of 80 to 100 mU. oxytocin (O) and less than 5 mU. vasopressin (V), so that the V/O ratio was approximately 1/20. The doses used in the present observations gave an arginine vasopressin/oxytocin ratio of 1/75 and a lysine vasopressin/oxytocin ratio of 1/20 or 1/30. In water diuresis 2 mU. arginine vasopressin and 7 mU. lysine vasopressin caused antidiuresis of equal degree, and this degree was not changed by the injection of oxytocin with the vasopressins; but the duration of the antidiuresis due to arginine vasopressin was shortened by the 75 times greater dose of oxytocin, whereas the already short antidiuresis caused by lysine vasopressin was unchanged by 20 times as much oxytocin. With regard to Na, at a low rate of urine flow both vasopressins showed antagonism to oxytocin, since when given together with oxytocin they cut short the increased Na loss caused by oxytocin alone. On the other hand, during water diuresis no antagonism appeared since a sharper, though not longer, rise in Na loss followed the injection of either vasopressin together with oxytocin. Since the vasopressins either fully or partially antagonized the action of oxytocin on glomerular filtration rate and renal plasma flow it is not possible to explain the enhanced Na loss in diuresis when both oxytocin and vasopressin were injected, as due to the specific action of vasopressin summed with an increase in filtered Na load. Obviously some other factor has to be considered, perhaps the rate of flow of tubular urine.

Regarding K excretion and comparing the two vasopressins from the standpoint of equi-antidiuretic doses, lysine vasopressin always increased the rate of loss of this ion at high or low rate of urine flow, whereas arginine vasopressin had little effect. When lysine vasopressin was given together with oxytocin there seemed to be a rather greater loss of K, but following arginine vasopressin plus oxytocin the loss of K was still small and inconstant. In general, then, as far as electrolyte excretion was concerned, the two vasopressins were alike in their behaviour towards Na, but lysine vasopressin had a greater power to raise the rate of K loss.

Measurements of glomerular filtration rate and renal plasma flow showed that 2 mU. of arginine vasopressin was able nearly completely to antagonize the effects of 150 mU. oxytocin on the renal blood vessels, that is, this small dose of arginine vasopressin was able almost to counteract 75 times as much oxytocin, or about twice the presumed physiological dose (Abrahams and Pickford, 1954). The dose of lysine vasopressin (7 mU.) equivalent in terms of antidiuresis to 2 mU. arginine vasopressin only partially antagonized the action of oxytocin on glomerular filtration rate and renal plasma flow, and for complete antagonism 10.5 mU. lysine vasopressin had to be used, that is, a lysine vasopressin/oxytocin ratio of 1/15. It seems, then, that although arginine vasopressin and lysine vasopressin are equally potent in raising the systemic blood pressure in anaesthetized dogs, lysine vasopressin has a feebler action than

arginine vasopressin on renal blood vessels dilated by oxytocin. The physiological significance of these results is uncertain since there is no direct evidence as to the variety of vasopressin occurring naturally in dogs, but from the results of their own observations van Dyke, Engel, and Adamsons (1956) concluded that it is arginine vasopressin.

The main pharmacological points emerging from these experiments are, first, that in the dog both arginine vasopressin and lysine vasopressin behave in parallel manner towards Na excretion, and somewhat alike towards water, though lysine vasopressin must be used in a larger dose. Second, that relative to its antidiuretic and Na excreting potency, lysine vasopressin is more active than arginine vasopressin in augmenting K loss ; third, arginine vasopressin, in the circumstances considered, is far more efficient than lysine vasopressin at restoring to normal glomerular filtration rates and renal plasma flows which have been raised by oxytocin. The potencies of the two vasopressins vary in such a manner that it is unlikely that their effects on Na and K excretion are related to their vascular activities.

The results offer further evidence that there can be independent regulation of the excretion of water, sodium, and potassium.

Grateful thanks are due to Dr. V. du Vigneaud and Dr. H. B. van Dyke for their generous supplies of purified natural vasopressin and oxytocin, and to Dr. Holgate of Messrs. Sandoz Ltd. for supplies of synthetic oxytocin. Sincere thanks are due to Dr. M. Pickford for valuable advice and suggestions and for operative preparation of the dogs. The work was undertaken during the tenure of a scholarship sponsored by the Government of Pakistan. This work was part of that presented in a thesis for the degree of Master of Science and accepted by the University of Edinburgh.

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Brit. J. Pharmacol. (1958), **13**, 138.

EFFECTS OF RATE OF STIMULATION AND OF FATIGUE ON THE RESPONSE TO NEUROMUSCULAR BLOCKING AGENTS

BY

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The response to neuromuscular blocking agents, as exemplified by suxamethonium and tubocurarine, may differ between symmetrical muscles. Increased rates of stimulation and fatigue both increase sensitivity to blocking agents. During recovery from suxamethonium block muscles may show a transitory failure to react to each stimulus with a contraction. Fatigue caused by prolonged indirect stimulation is prevented equally well by suxamethonium and tubocurarine in doses producing a complete block. The mechanism and significance of these findings are discussed.

Sensitivity to neuromuscular blocking agents varies between different muscles ; for instance, the external muscles of the eye are paralysed by smaller doses than are necessary to paralyse the muscles of the limbs (Paton, 1953). These disparities may be related to the frequency of the impulses which the various muscles receive *in vivo* from the central nervous system. This rate is much higher in the external ocular muscles than in those of the extremities (Denny-Brown, 1929 ; Adrian and Bronk, 1929 ; Cooper and Eccles, 1930). Quantitative studies on the influence of rate of stimulation on neuromuscular block have been made in the rat phrenic nerve-diaphragm preparation (Chou, 1947), in dogs by means of electromyograms (Guyton and Reeder, 1949), in cats and dogs with regard to respiratory depression (Li, Jacobs, Aviado, and Schmidt, 1952), and in the rat sciatic nerve-gastrocnemius preparation (Preston and Maanen, 1953). All these investigations showed that increased frequency of stimulation resulted in a decrease in the amount of drug required to produce the same degree of block.

The response to blocking agents may also be altered by fatigue because during fatigue, as in partial block, the twitch tension of the muscle is diminished (Gilson, Schoepfle, and Walker, 1947 ; Brown and Burns, 1949). Symptoms resembling those that follow violent exercise may appear in man after an injection of suxamethonium though not after tubocurarine (Churchill-Davidson, 1954 ; Price, 1954).

The experiments described in this paper, some of which have been briefly reported (Wislicki, 1956), concern the influence exerted by variations in the rate of stimulation and by fatigue on the paralysis caused by suxamethonium and tubocurarine. The effect of prolonged block on the response of the muscle to a further dose of the same drug was also investigated.

METHODS

Anaesthesia in cats was induced with ether and maintained with intravenous pentobarbitone sodium. A carotid artery was cannulated for blood pressure measurements and a femoral vein for injections. Respiration was recorded by means of a tambour connected to a tracheal cannula whose side arm was fitted with an adjustable leak.

The tendon of each gastrocnemius was attached to an isometric lever. Both sciatic nerves were divided high in the thigh and the peripheral ends placed on shielded silver electrodes, the proximal electrode being the anode. Repetitive supramaximal rectangular stimuli of 0.1 msec. duration were applied from a Fleming stimulator. When both sides were stimulated simultaneously at the same frequency the two pairs of electrodes were connected to one channel. For the experiments where the rates of stimulation were different for each leg the two channels were separated by placing a Muirhead D-106-A/1 transformer between each channel and its electrodes, for which arrangement I am indebted to Mr. J. Weinman.

Suxamethonium chloride and tubocurarine chloride were the blocking agents used. Doses are given in terms of these salts. In those animals which received

several injections, only small doses were administered and intervals of at least 15 min. for suxamethonium and 50 min. for tubocurarine were allowed between injections to avoid accumulation, except in those experiments in which a prolonged complete block was required.

RESULTS

When stimulation was applied to both legs simultaneously at the same frequency, the response to blocking agents often differed between the two sides (Fig. 1).

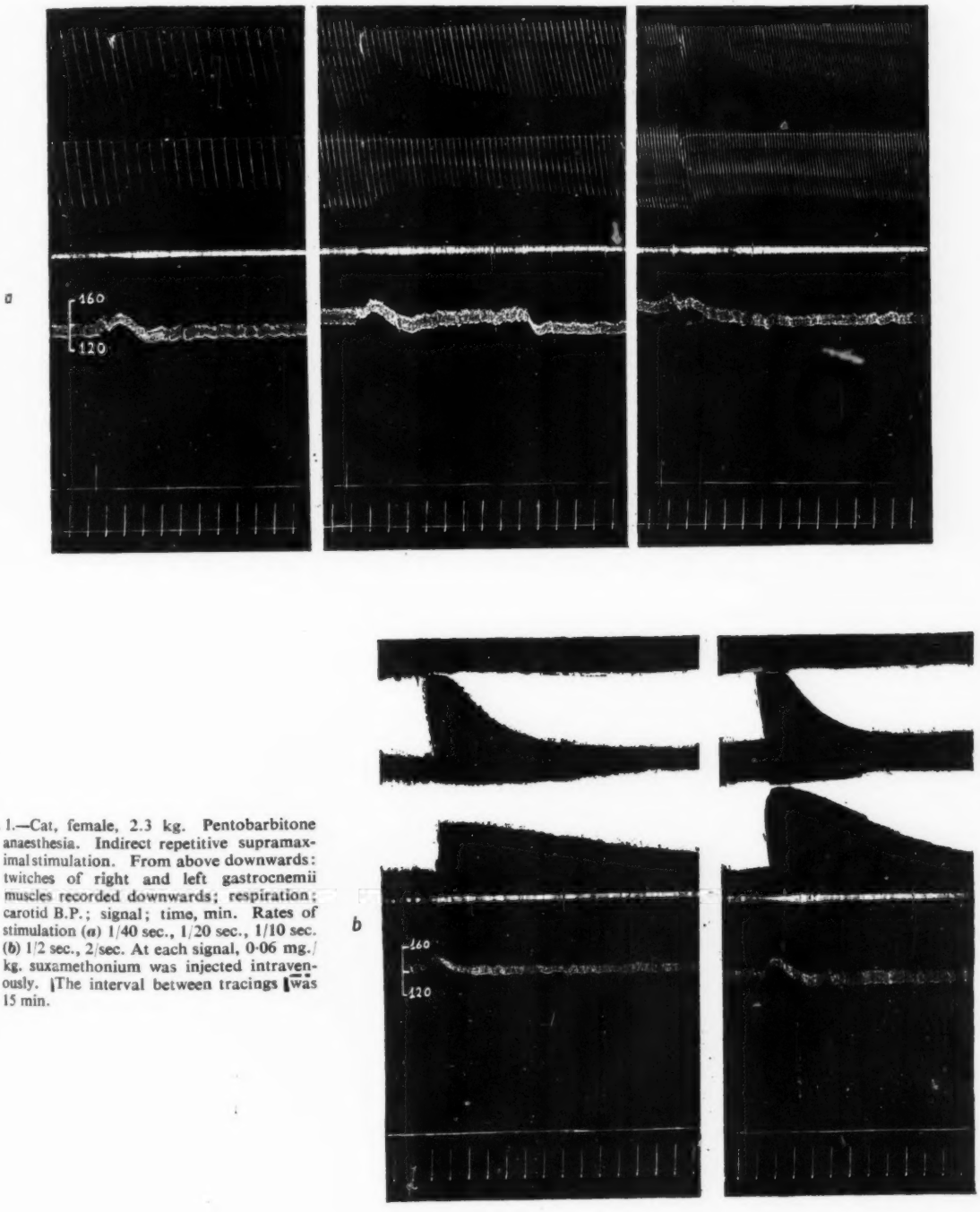


FIG. 1.—Cat, female, 2.3 kg. Pentobarbitone anaesthesia. Indirect repetitive supramaximal stimulation. From above downwards: twitches of right and left gastrocnemii muscles recorded downwards; respiration; carotid B.P.; signal; time, min. Rates of stimulation (*a*) 1/40 sec., 1/20 sec., 1/10 sec. (*b*) 1/2 sec., 2/sec. At each signal, 0·06 mg./kg. suxamethonium was injected intravenously. The interval between tracings was 15 min.

Disparities could be seen with any frequency of stimulation but were usually more striking at relatively high frequencies, such as 1/2 sec. in Fig. 1. The difference in sensitivity between the two sides, measured as the ratio of equi-active doses, might be as great as 1.5:1.

Fig. 1 also shows that the reaction to suxamethonium depended on the rate of stimulation. At 1/40 sec. 0.06 mg./kg. depressed contractions only slightly, but the effect of the same dose was much greater at higher frequencies. Transmission in the more sensitive gastrocnemius was completely blocked at 1/2 sec. and in the other muscle at 2/sec. To produce a block of a certain intensity during repetitive stimulation at 1/40 sec. about twice the dose of suxemethonium was required as at 1/sec. and 3 to 5 times the amount as during subtetanic stimulation. At rates of less than 1/30 sec. the effect of a dose of a blocking agent varied very little (25 animals).

The response to tubocurarine varied with the frequency of stimulation in the same manner (Fig. 2).

When during subtetanic stimulation a transient block was produced by a small dose of suxamethonium, the first contractions to appear during recovery often had a frequency lower than the rate of stimulation. The ratio between the two frequencies varied in different experiments. In the experiment illustrated in Fig. 3 a frequency of 15 shocks/sec. caused a subtetanus which was blocked by 0.06 mg./kg. suxamethonium. Within 2 min. contractions returned at a rate of 8/sec., gradually increasing in size for about half a min. The twitches then became more frequent but smaller, and subtetanus reappeared.

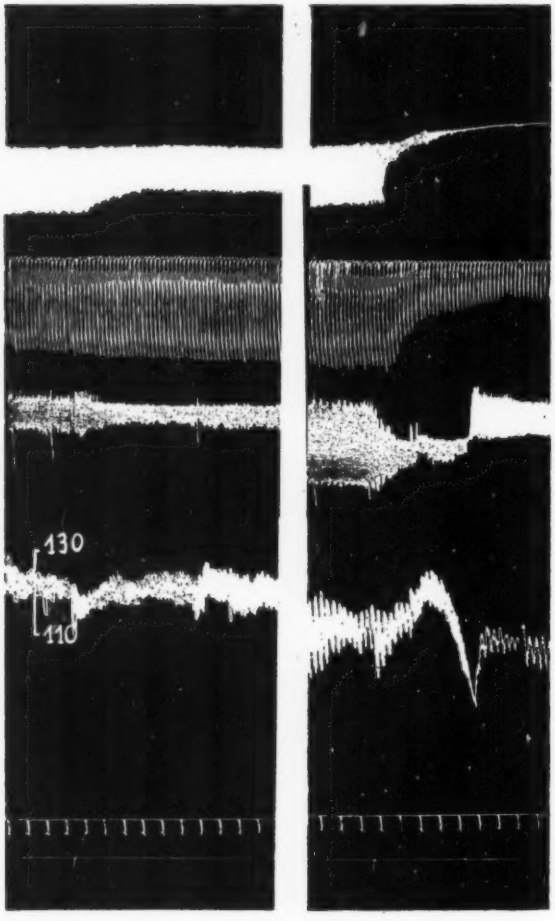


FIG. 2.—Cat, male, 2.9 kg. Pentobarbitone anaesthesia. Recordings as in Fig. 1. Towards the end of (*b*) artificial respiration was given. Rates of stimulation: upper muscle record, 1/sec.; lower muscle record, 1/10 sec. At the signals tubocurarine was given intravenously, at (*a*) 0.15 mg./kg., and at (*b*) 0.25 mg./kg. The interval between tracings was 50 min. Time, 1 min.

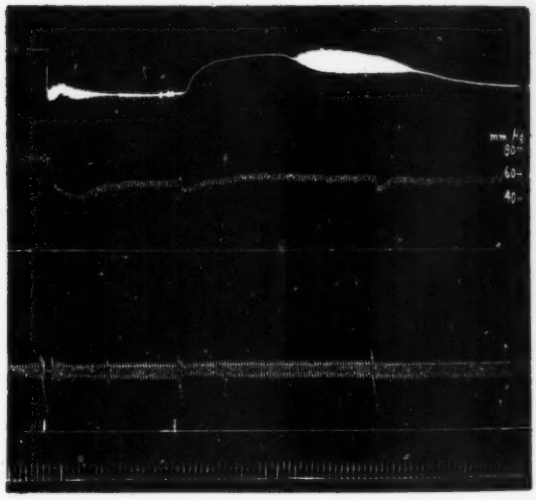


FIG. 3.—Cat, male, 3.2 kg. Pentobarbitone anaesthesia. Indirect supramaximal stimulation at 15/sec. From above downwards: gastrocnemius twitch; blood pressure; zero line for blood pressure; respiration; signal; time, 5 sec. At the first signal stimulation was started and at the second 0.06 mg./kg. suxamethonium was injected intravenously.

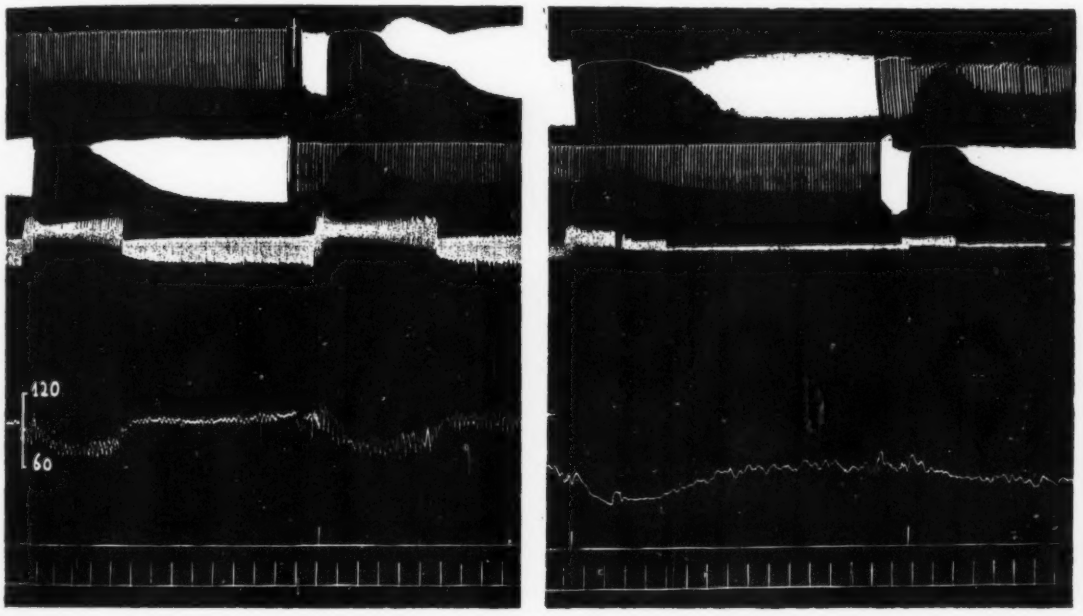
This transient discrepancy in frequencies of stimulation and response was not observed after large doses of suxamethonium given during subtetanic stimulation or with small amounts injected during tetanic contraction.

The influence of muscle fatigue on the effect of blocking agents was studied by testing the response of the same muscle after prolonged stimulation at high and low frequencies (Fig. 4). For 45 min. one gastrocnemius was stimulated at 1/10 sec., the other at 3/sec. Neither muscle showed signs of

fatigue, the twitch height remaining constant. Then 0.75 mg./kg. suxamethonium was injected while stimulation continued at the same frequencies. In the muscle stimulated at 1/10 sec. the response was very slight while in that stimulated at 3/sec. transmission ceased for 3 min. The channels of the stimulator were then switched so that the muscle which had been stimulated at 1/10 sec. was now stimulated at 3/sec. and *vice versa.* After a few minutes, the same dose of suxamethonium now caused complete block in both muscles (Fig. 4*a*).

exclude this possibility the new rates of stimulation were maintained for an hour and the same dose of suxamethonium was injected again. It now caused a complete block in the muscle stimulated rapidly and only a partial block in the muscle stimulated slowly (Fig. 4*b*). The stimulation was again reversed and, within a few minutes, a further dose of suxamethonium produced complete block of both muscles.

This result was confirmed in 8 similar experiments. The reaction of the muscle to the action of suxamethonium was always intensified after



a *b*

FIG. 4.—Cat, female, 3.5 kg. Recording arrangement as in Fig. 1. Periods of artificial respiration shown as elevations on respiratory tracings. At signals, 0.75 mg./kg. of suxamethonium was injected intravenously. Time, 1 min. See also table below this legend.

Muscle Recording	Rates of Stimulation			
	During 45 min. Prior to (*a*)	(*a*)	During interval of 55 min. between (*a*) and (*b*)	(*b*)
Upper ..	1/10 sec.	1/10 sec., later 3/sec.	3/sec.	3/sec., later 1/10 sec.
Lower ..	3 sec.	3/sec., later 1/10 sec.	1/10 sec.	1/10 sec., later 3/sec.

This increased response during stimulation at low frequency in the muscle previously subjected to high frequency stimulation for 45 min. might conceivably have been due to an initial difference in sensitivity between the two gastrocnemii. To

prolonged rapid stimulation. This effect was reversible provided that the twitch tension remained unchanged throughout, indicating that only a slight degree of fatigue was present.

The effect of a blocking agent on a severely fatigued preparation is recorded in Fig. 5. The gastrocnemius of one leg was allowed to rest while the other was stimulated at 4/sec. for 2 hr., thereby reducing the twitches to a fraction of their initial height. Both muscles were then stimulated at 1/10 sec. for 5 min., and 0.05 mg./kg. tubocurarine was injected. This had hardly any effect on the rested muscle, but in the fatigued one a complete block developed. Moreover, the preparation did not recover completely since the twitch tension remained smaller than it had been before the injection of tubocurarine.

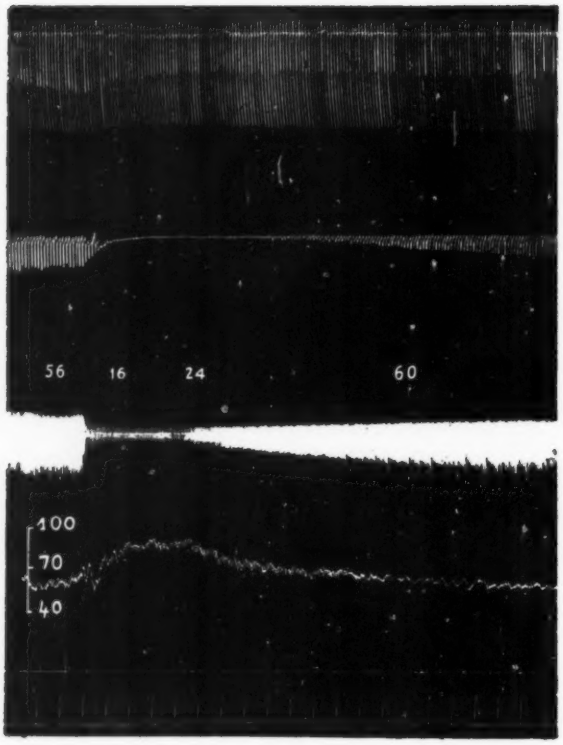


FIG. 5.—Cat, female, 3.5 kg. Recording arrangement as in Fig. 1. The muscle recorded at the top was after rest, but the lower muscle recording was after stimulation at 4/sec. for 120 min. The numerals above the respiratory tracing signify respiration rate. At the signal 0.05 mg./kg. tubocurarine was injected intravenously. Time, 1 min.

It seemed possible that the block produced by suxamethonium might be accompanied by changes of the neuromuscular junction comparable to those occurring during fatigue. This was tested in the following way. A gastrocnemius was stimulated at 2/sec. and its response to the test dose of either suxamethonium or tubocurarine was ascertained.

The frequency was then increased to 4/sec., and, with stimulation continuing all the time, the same blocking agent was administered in very large amounts: 1 mg./kg. suxamethonium or 0.4 mg./kg. tubocurarine was given repeatedly as required to maintain a complete block for 2 hr. Stimulation was then switched back to 2/sec., and when the preparation had recovered from the block the same test dose as at the beginning was administered again. With both suxamethonium and tubocurarine the reaction to this last injection was found to be the same as to the initial dose, indicating that the muscle had not been affected by the fast stimulation during the long period when transmission was blocked. Hence, no evidence was found for the assumption that the block produced by suxamethonium is accompanied by changes at the neuromuscular junction similar to those produced by fatigue.

DISCUSSION

The alterations in the effects of suxamethonium and tubocurarine with variations in the frequency of stimulation appear to be due to an effect of repetitive stimulation on the events which take place at the neuromuscular junction. Although the local and propagated potential changes last for only a few msec. they may be influenced by events which occur at much slower rhythms. Eccles, Katz, and Kuffler (1941) reported that after two closely spaced nerve volleys the end-plate potential did not recover for several seconds. Likewise, in the lightly curarized preparation, a brief tetanus is followed by electrical changes which may last for more than 98 sec. (Liley and North, 1953). Thus repetitive stimulation at intervals from 40 to less than 0.5 sec., as applied in our experiments, could have an increasing effect at the neuromuscular junction and thereby vary the response to blocking agents. By analogy, the higher the rate of impulses a muscle receives from the central nervous system the more sensitive it will be to such drugs.

Blood flow is another factor which, *in vivo*, determines the speed of development and the depth of a neuromuscular block. During rhythmic exercise blood flow is increased (Barcroft and Swan, 1953). Accordingly, the higher the rate of stimulation the greater will be the increase in blood flow and the widening of the vascular bed through the opening-up of more capillaries. Therefore, after intravenous injection of a blocking agent, its peak concentration in the tissues will occur earlier and will be spread over a greater number of neuromuscular junctions, the faster the rate of stimulation. Block will thus develop more quickly and to a greater degree. Variations of blood flow, although contributive, cannot, however, be decisive for the increase in the sensitivity to blocking agents observed with faster stimulation, since the same dependence has been found *in vitro* in the rat phrenic nerve-diaphragm preparation (Chou, 1947) where differences in blood flow do not apply.

Muscles under subtetanic stimulation, when recovering from block due to a small dose of suxamethonium, showed a transitory failure to contract after each impulse. Brown and Burns (1949) demonstrated, in the fatigued muscle, the presence of a neuromuscular block. They found

that muscle fibres to which transmission was failing responded to some, but not to all, nerve impulses. Here there is an apparent similarity between the effect of neuromuscular blocking agents and fatigue.

On the other hand, the effect of blocking drugs is intensified after exercise. This intensification precedes any diminution in twitch tension, for it occurs at a time when fatigue of the neuromuscular junction only can be involved. In fatigue, the decrease of twitch tension is not due to a depression of neuromuscular transmission but to a decline in contraction strength as the biochemistry of the contractile process becomes defective (Merton, 1954; Edwards and Lippold, 1956). This mechanism clearly differs from that of blocking drugs which reduce the twitch tension during partial block by preventing some of the muscle fibres from responding to stimulation.

I am indebted to Messrs. Allen and Hanburys Ltd. for a supply of "scoline."

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METABOLIC FACTORS AND VENTRICULAR FIBRILLATION

BY

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Ventricular fibrillation has been studied in the isolated rabbit heart to determine the effect of factors modifying the metabolism. Sodium azide, sodium monoiodoacetate and sodium fluoride were found to cause fibrillation, the effect of sodium fluoride being neutralized by magnesium chloride. Fibrillation was also caused by lack of glucose and could be arrested by adding glucose; the effect of glucose in arresting fibrillation was facilitated by insulin. In other experiments mannose and pyruvate could arrest fibrillation due to lack of glucose, but L(–)lactate could not. The effect of temperature changes, of adrenaline and of cyanide were also studied. When all oxygen was removed from the perfusing solution fibrillation was arrested.

The belief is widely held that ventricular fibrillation occurs in circumstances in which there is interference with normal metabolic processes, and that it is in some way a consequence of a failure of the sources of energy which are ordinarily at the disposal of cardiac muscle. Investigation of this idea when fibrillation occurs in the animal is difficult because fibrillation is usually irreversible and because it is impossible to distinguish between primary and secondary factors.

A method is now available for the study of ventricular fibrillation in the isolated heart of the rabbit. The heart is perfused through the aorta by the method of Langendorff and electrical stimulation is applied to the ventricle at a high rate. Fibrillation then occurs. When the stimulation is stopped the fibrillation either reverts very soon to a normal rhythm or else it persists, according to the composition of the fluid perfusing the heart (Armitage, Burn, and Gunning, 1957).

This method has now been used to investigate the effect of metabolic inhibitors, and of other factors affecting metabolism such as the presence of adrenaline, temperature, changes in oxygen supply and absence of glucose. In the work quoted the effect of 2-4-dinitrophenol was investigated; when present in the perfusion fluid in concentrations 3 to 5×10^{-6} molar, this substance caused fibrillation for long periods.

METHODS

The normal perfusion fluid contained NaCl 7.7 g., KCl 0.42 g., $CaCl_2$ 0.24 g., $NaH_2PO_4,2H_2O$ 0.14 g., $NaHCO_3$ 2.1 g., dextrose 2 g., sucrose 4.5 g., distilled water to 1 l. The solution was saturated with 95% oxygen and 5% CO_2. The temperature was maintained within $\pm 0.5°$ using the device of Saxby (1956). The pressure head at which the hearts were perfused was 54 cm. water. The ventricles were stimulated by platinum fish-hook electrodes, one piercing the wall of the left ventricle at the apex and the other near the base midway between the coronary arteries. The stimuli were 1 mA. in strength and 0.75 msec. in duration; they were applied usually at the rate of 10/sec. but always at the same rate in any one experiment. When stimulation was not being applied the electrodes were used as leads to an electrocardiograph (Cossor model 1314) on which the rhythm was recorded.

In testing substances to see if they would cause fibrillation, use was made of the finding by Armitage *et al.* (1957) that the occurrence of fibrillation as a result of stimulation depended on the concentration of K^+ in the perfusing fluid. In some hearts persistent fibrillation (which in practice was taken to be fibrillation lasting for 15 minutes) followed electrical stimulation when the perfusion fluid contained the normal concentration of K^+, namely 5.6 mM. This fibrillation was arrested by pouring cold saline over the heart (Dirken, Gevers, Heemstra, and Huizing, 1955). If the perfusion fluid was changed to one containing a higher concentration of K^+ such as 7 mM., stimulation then usually failed to produce more than a transient fibrillation (less than 5 minutes), a normal rhythm returning spontaneously; if stimulation still caused persistent fibrillation, the K^+ concentration was increased further to 8.4 mM. Conversely if stimulation during perfusion with a solution containing K^+ in concentration 5.6 mM. failed to cause more than transient fibrillation, the heart was perfused with a lower concentration of K^+ in the expectation that stimulation would then cause persistent fibrillation.

Thus at the beginning of each experiment a concentration of K^+ was determined in which stimulation caused only a transient fibrillation and a lower con-

centration of K^+ was also determined in which stimulation caused persistent fibrillation. The substance which was to be tested was then added to the solution containing the higher concentration of K^+, and after this solution had been perfused through the heart for 20 to 30 minutes stimulation was applied to determine the effect.

When the composition of the perfusing solution was modified by the inclusion of a substance, such as mannose, in sufficient amount to alter the tonicity of the solution, the sucrose concentration was adjusted accordingly.

Results

Sodium Azide.—Results with sodium azide are set out in Table I, which shows the duration of fibrillation after stimulation in 6 experiments. In expt. 1, stimulation during perfusion without sodium azide caused fibrillation for 1.5 min., when a normal rhythm returned. Sodium azide in a concentration 3×10^{-3} M was then added to the same perfusion fluid, and, after the perfusion had continued for 25 min., stimulation was applied. Fibrillation occurred and continued for 19 min. after stimulation before reverting to a normal rhythm. The perfusion was then continued for 20 min. without sodium azide, and when stimulation was applied there was no period of fibrillation afterwards.

TABLE I

EFFECT OF SODIUM AZIDE (3×10^{-3}M) IN CAUSING FIBRILLATION

An asterisk indicates that fibrillation was arrested by cooling.

Expt. No.	Duration of Fibrillation after Stimulation (min.)		
	Before Azide	In Presence of Azide	After Removing Azide
1	1·5	19	0
2	0	54	7
3	0	27	0
4	9	22*	0
5	1	29*	0
6	0	6	0

The results made it clear that fibrillation occurred more readily and for a longer time when the metabolism was depressed by the presence of sodium azide, the change being fully reversible when the sodium azide was removed.

Sodium Monoiodoacetate. — The effect of sodium monoiodoacetate on fibrillation was more difficult to determine than that of sodium azide, since it had no action until nearly toxic concentrations were used, when its action was not readily reversible. The concentrations used were 2.1 or 3.2×10^{-5} M. The results in Table II show that the presence of sodium monoiodoacetate caused fibrillation to follow stimulation in 7 out of 12 hearts. In the remaining 5 hearts, there was no evidence that monoiodoacetate interfered with metabolism sufficiently to cause fibrillation.

TABLE II

EFFECT OF SODIUM MONOIODOACETATE (IAA)

An asterisk indicates that fibrillation was arrested by cooling.

Expt. No.	Duration of Fibrillation after Stimulation (min.)		Molar Concentration of IAA
	Before IAA	In Presence of IAA	
7	0	13*	$3·2 \times 10^{-5}$
8	0	5*	$3·2 \times 10^{-5}$
9	0	6*	$3·2 \times 10^{-5}$
10	0	6*	$3·2 \times 10^{-5}$
11	0	4·5*	$3·2 \times 10^{-5}$
12	0	0	$3·2 \times 10^{-5}$
13	0	0	$3·2 \times 10^{-5}$
14	0	31	$2·1 \times 10^{-5}$
15	0	0	$2·1 \times 10^{-5}$
16	0	18	$2·1 \times 10^{-5}$
17	0	0	$3·2 \times 10^{-5}$
18	3	0	$3·2 \times 10^{-5}$

Sodium Fluoride.—The action of sodium fluoride was much clearer than that of sodium monoiodoacetate, for it increased the duration of fibrillation in all trials, as shown in Table III.

TABLE III

EFFECT OF FLUORIDE ($2·5 \times 10^{-4}$M) ON FIBRILLATION

An asterisk indicates that fibrillation was arrested by cooling.

Expt. No.	Duration of Fibrillation after Stimulation		Time Required for $MgCl_2$ to Arrest Fibrillation (min.)	Molar Concentration Mg++
	Before Fluoride (min.)	In Presence of Fluoride (min.)		
19	0	63	Not arrested	2×10^{-4}
20	0	16*	—	—
	0	10*	—	—
21	0	9*	—	—
	7	15	0·5	2×10^{-4}
	—	11	0·5	2×10^{-4}
22	—	13	1·0	10^{-5}
23	0	6*	—	—
	—	43	0·5	10^{-5}
	—	30	Not arrested	10^{-6}

The effect of fluoride was reversible, and moreover the fibrillation induced in its presence was promptly arrested by magnesium chloride. Thus in expt. 21 there was no fibrillation after stimulation in the absence of fluoride, but in its presence there was fibrillation for 9 min.; this was arrested by cooling the heart. The heart was again perfused without fluoride, and on stimulation there was fibrillation for 7 min. which reverted to normal rhythm spontaneously. The heart was then perfused with fluoride and after stimulation fibrillated for 15 min. The perfusion fluid was changed to one containing $MgCl_2$ as well as fluoride, and fibrillation was arrested in 0.5 min. The heart was again perfused with fluoride without $MgCl_2$, and after stimulation it fibrillated for

11 min. On changing to perfusion with fluoride and $MgCl_2$, the fibrillation was again arrested in 0.5 min.

Lack of Glucose.—Since it is known that glycolysis is inhibited by fluoride and that this inhibition is neutralized by magnesium, the foregoing results led to the investigation of the effect of a lack of glucose. In 4 out of 5 hearts it was found that perfusion with a glucose-free solution for 30 min. made no difference to the effect of stimulation, but in one heart persistent fibrillation was observed. Experiments were therefore carried out in which the perfusion fluid was without glucose for a longer time, and in these it was found that the lack of glucose always caused fibrillation after stimulation, and that fibrillation due to the absence of glucose was usually arrested by adding glucose. The following experiment provides an example. A heart perfused with glucose-free solution was stimulated for 2 min. and fibrillation was produced. After 5 min. this fibrillation was arrested by cooling the ventricles. The K^+ concentration was then raised from 5.6 to 7.0 mM., and 12 min. later stimulation was repeated. Fibrillation did not occur. Nor did it occur when stimulation was repeated after a further 15 min. However, when the heart was stimulated 20 min. later still, by which time it had been perfused with a glucose-free solution for 70 min., fibrillation began and continued for 39 min. At this point perfusion was continued with a fluid containing 11 mM./l. glucose, and after 4 min. fibrillation stopped and a normal beat was recorded on the drum and on the electrocardiograph.

The results of this series of experiments are given in Table IV. In expts. 24 (see Fig. 1), 31 and 34 fibrillation occurred spontaneously although the K^+ concentrations were 8.4, 7.7, and 7.7 mM.

TABLE IV

EFFECT OF GLUCOSE-FREE SOLUTION IN CAUSING FIBRILLATION

An asterisk indicates that fibrillation occurred spontaneously.

Expt. No.	Time of Perfusion with Glucose-free Solution before Stimulation (min.)	Duration of Fibrillation after Stimulation (min.)	Time Taken for Arrest of Fibrillation by Glucose (min.)
24	99*	12	2
25	104	16	9
26	145	18	8
27	85	15	4
28	77	17	4
29	105	38	0·5
30	70	39	4
31	48*	34	(No arrest)
32	55	35	4
33	160	39	1
34	43*	37	(No arrest)

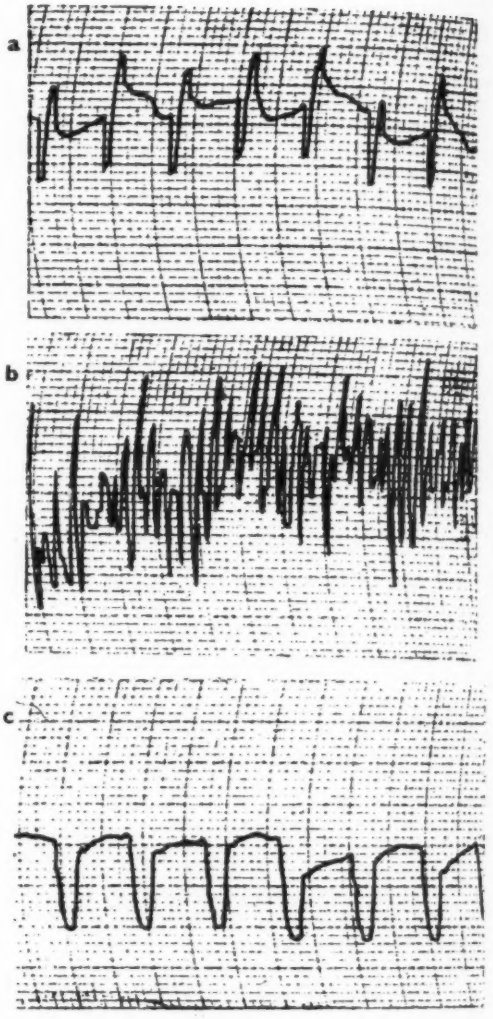

FIG. 1.—Electrocardiograms recorded from the isolated rabbit heart. (a) Shows the regular rhythm when the heart was first perfused with glucose-free solution. (b) Shows spontaneous fibrillation. (c) Shows the restoration of regular rhythm on changing to a solution containing glucose.

respectively. Spontaneous fibrillation had otherwise only been observed when the K^+ concentration was as low as 1.4 mM. In expts. 31 and 34 readmission of glucose did not arrest the fibrillation.

Insulin and Glucose.—Since insulin facilitates the entry of glucose into the cells (see Stadie, 1954), observations were made with glucose and insulin. In 3 out of 4 experiments evidence was obtained that the addition of insulin arrested fibrillation when the heart had been perfused with a low-glucose solution for some time. In one experiment the heart was perfused with $\frac{1}{8}$ normal

glucose, that is with 1.4 mM. for 175 min., and stimulation then produced fibrillation which was allowed to continue for 34 min. The perfusate was changed to one also containing insulin in a concentration of 2 units/l. The normal rhythm returned in 2 min. The results in a second experiment were similar, the time of perfusion before stimulation being 145 min., the time of fibrillation after stimulation being 31 min. and the normal rhythm returning on addition of insulin after 10 min. In a third experiment, however, the addition of insulin failed to stop the fibrillation. In a fourth experiment fibrillation was produced in a glucose-free medium and observed for 38 min. The addition of glucose (11 mM.) failed to stop this fibrillation during 11 min., but when 1 unit of insulin was injected the fibrillation stopped in 1 min.

Substitutes for Glucose.—Observations were next made to see whether the fibrillation produced by stimulation in a glucose-free medium could be arrested by substances other than glucose. The first to be tried was mannose. When added to the normal fluid perfusing a heart, mannose in 11 mM. concentration caused a progressive diminution of the beat; this diminution was still more rapid after removing the glucose. The amplitude was not restored by readmitting glucose as always happened in the absence of mannose. In spite of this effect on the heart, mannose arrested fibrillation in a glucose-free medium in 3 out of 6 hearts. Of the 3 hearts in which mannose failed to arrest fibrillation the addition of glucose was effective in one.

Observations were also made with pyruvate. When fibrillation was produced by stimulation in a glucose-free medium, the addition of sodium pyruvate to the perfusion fluid arrested fibrillation in one heart and converted fibrillation to a tachycardia in a second; in 4 hearts it had no effect. However, fibrillation which was produced as a result of stimulation in a glucose-free medium, could not be produced in the presence of pyruvate in 3 hearts. The evidence indicated that pyruvate had some effect, but was less effective than glucose.

Observations were also made with lactate. I am grateful to Professor H. A. Krebs for supplying me with a sample of pure L(+)lactic acid free from the D-isomer, from which sodium L(−) lactate was prepared. This was tested in 5 hearts; each heart was fibrillating in glucose-free perfusate, and the addition of sodium L(−)lactate to the perfusate so that the concentration was 22 mM. did not arrest the fibrillation. In 2 of these hearts the fibrillation was arrested later by the addition of glucose and in another by the addition of glucose and insulin. Thus no evidence was obtained that L(−)lactate was effective in arresting the fibrillation.

The Effect of Temperature.—While the foregoing results were consistent with the view that fibrillation occurs in circumstances in which metabolism is disturbed, some observations are now described which at first sight appear to conflict with this view. That they may not do so will be considered in the discussion.

Observations on the effect of temperature were made in 10 hearts and the results are illustrated in Table V. The procedure in expt. 35 was to stimulate the heart electrically for 1 min. at 37° when the perfusion fluid contained 5.6 mM. K^+. After the stimulation stopped the ventricles did not fibrillate, and the beat was regular. The K^+ concentration in the perfusion fluid was then reduced to 4.2 mM., and after 20 min. stimulation was repeated. When the stimulation was stopped, the ventricles fibrillated for 6 min. and at this point the temperature of the perfusion fluid was lowered to 32°. After 5 min. more the fibrillation ceased, having continued for 11 min. When stimulation was applied for as long as 10 min. at this lower temperature fibrillation did not occur. The temperature was then raised once more to 37°, and stimulation for 1 min. caused fibrillation which was observed for 36 min. Similar results were obtained in the other experiments shown in Table V, and in 6 experiments not shown. Thus under conditions in which fibrillation occurred at 37° it did not occur at 32°.

TABLE V

EFFECT OF TEMPERATURE ON FIBRILLATION

An asterisk indicates that fibrillation was arrested by cooling.

Expt. No.	K^+ Concentration (mM.)	Temp.	Duration of Fibrillation after Stimulation (min.)
35	5·6	37	0
	4·2	37	11 (see text)
	4·2	32	0
	4·2	37	36*
36	5·6	37	0
	4·2	37	29*
		32	0
		37	11*
		32	2
		37	20*
37	4·9	37	13*
		32	0
		37	13*
38	5·6	37	38*
		27	0
		32	0
		37	42*
		27	0·25
		37	30*

The Effect of Adrenaline.—Since the explanation of observations with adrenaline may be related to that of the foregoing observations on temperature they are described at this point. Each heart was initially perfused with a fluid containing normal K^+ (5.6 mM.) and was stimulated electrically. The course of one experiment is shown in Table VI in which this stimulation caused the ventricles to fibrillate for 9 min.; at this time a regular beat was restored by pouring cold saline over the heart. The K^+ concentration was then raised first to 7.0 and then to 7.7 mM., at which concentration the heart did not fibrillate on stimulation. The fluid was next changed to one containing adrenaline (4×10^{-7} g./ml.), and with this perfusate stimulation caused fibrillation. Five further trials were made in which adrenaline was alternately absent and present, and, as the results in Table VI show, fibrillation did not occur when it was absent, but always when it was present. Similar results were obtained in 7 out of 8 hearts.

TABLE VI

EFFECT OF ADRENALINE ON FIBRILLATION

An asterisk indicates that fibrillation was arrested by cooling.

Expt. No.	K^+ Concentration (mM.)	Adrenaline Concentration (g./ml.)	Duration of Fibrillation after Stimulation (min.)
39	5·6	—	9*
	7·0	—	1
	7·7	—	0
	7·7	4×10^{-7}	9*
	7·7	—	0
	7·7	4×10^{-7}	4*
	7·7	—	0
	7·7	4×10^{-7}	9*
	7·7	—	0

The Effect of Oxygen Lack.—Since lack of glucose was found to lead to fibrillation, the effect of lack of oxygen was also studied. Experiments were therefore carried out in which the perfusion fluids were prepared from well-boiled water and were aerated with nitrogen and 5% CO_2. When hearts were perfused with this solution stimulation did not cause fibrillation, but on the contrary fibrillation was arrested.

The results of a series of experiments are given in Table VII in which fibrillation was produced during aeration with oxygen. This was allowed to continue for varying lengths of time as shown in the second column. The perfusion fluid was then changed to one aerated with nitrogen or to one aerated with 90% N_2 + 10% O_2 as shown in the third column. In each of the eight experiments in which the perfusion fluid was changed to one which was O_2-free a normal rhythm was restored, though a period as long as 16 min. was required in expt. 46. When, however, as little as 10% oxygen was present, fibrillation was arrested in only 2 out of 6 hearts. Thus the effect of oxygen lack in arresting fibrillation was seen only when the lack was complete. For example, in carrying out expt. 44 the ventricles were caused to fibrillate when the fluid was aerated with oxygen and continued to do so for 23 min. The aerating gas was then changed to one containing 10% O_2; this had no effect, the fibrillation continuing for the next 20 min. Then an O_2-free solution was perfused, and after 8 min. the fibrillation was arrested. Again in expt. 45, after fibrillation in O_2 for 15 min., the fluid was changed to one which was O_2-free. The fibrillation stopped immediately. Fibrillation was again induced in O_2 and continued for 17 min.; this time the gas mixture was changed to 10% O_2, but the fibrillation was unaffected during the next 10 min.

TABLE VII

EFFECT OF OXYGEN-FREE SOLUTION IN ARRESTING FIBRILLATION

Expt. No.	Period of Fibrillation in Presence of Oxygen (min.)	Gas Mixture Used to Replace Oxygen	Time before Regular Rhythm was Restored (min.)	Size of Beat after Fibrillation was Stopped (mm.)
40	17	N_2	2	0
41	16	N_2	10	3
42	19	N_2	11	—
43	16	N_2	13	—
44	23	90% N_2 + 10% O_2	(Not restored in 20)	
	(23 + 20)	N_2	8	0
45	15	N_2	0·5	40
	17	90% N_2 + 10% O_2	(Not restored in 10)	—
46	24	90% N_2 + 10% O_2	(Not restored in 20)	—
	(24 + 20)	N_2	16	0
47	22	90% N_2 + 10% O_2	(Not restored in 16)	—
	(22 + 16)	N_2	10	0
48	20	90% N_2 + 10% O_2	3	0
49	24	90% N_2 + 10% O_2	0	0

One comment on these results should be made. When fibrillation was arrested by other means, the change in the electrocardiogram was accompanied by the restoration of mechanical beats. The arrest of fibrillation by an O_2-free perfusate was not accompanied by a return of the contractions in 6 out of the 8 experiments.

The Effect of Sodium Cyanide.—The description of observations on sodium cyanide now follows because their complexity may appear less difficult to understand in the light of the observations on lack of oxygen. More experiments were made with sodium cyanide than with any other metabolic inhibitor because it was difficult to be certain what the effect was. There were experi-

TABLE VIII

EFFECT OF CYANIDE IN CAUSING FIBRILLATION

Expt. No.	Duration of Fibrillation after Stimulation (min.)		
	Before Cyanide	In Presence of Cyanide	Molar Concentration of Cyanide
50	0	30	10^{-4}
51	0	19	8×10^{-4}
52	0	18	3×10^{-4}
53	0	16	3×10^{-4}
	4	18	3×10^{-4}
54	0	20	3×10^{-4}
55	0	16	3×10^{-4}

ments in which it seemed clear that sodium cyanide behaved like sodium azide and sodium fluoride and led to the occurrence of prolonged fibrillation. Such results are set out in Table VIII. They were obtained with concentrations of cyanide from 10^{-4} M to 8×10^{-4} M. Expt. 53 gave a very clear picture, for the effect of stimulation was twice determined in the presence of cyanide and twice in its absence, whilst in expts. 52 and 54 the irregularity stopped as soon as the cyanide was removed.

There were also other experiments in which the presence of sodium cyanide arrested fibrillation as did perfusion with an O_2-free solution. These results are set out in Table IX in which are given

TABLE IX

EFFECT OF CYANIDE IN ARRESTING FIBRILLATION

An asterisk indicates that fibrillation was arrested by cooling.

Expt. No.	Duration of Fibrillation before Addition of Cyanide (min.)	Cyanide (Molar Concentration)	Time to Arrest Fibrillation (min.)	Amplitude of Restored Beat (mm.)
56	31	6×10^{-4}	13	0
57	18	3×10^{-4}	1	10
58	17	3×10^{-4}	5	10
	17	3×10^{-4}	17*	—
59	19	3×10^{-4}	1·5	50
	18	3×10^{-4}	6	1
60	17	3×10^{-4}	17*	—

the results of 7 trials on 5 hearts. In 5 of the 7 trials the addition of cyanide to the perfusion fluid arrested fibrillation which had persisted for periods varying from 17 to 31 min. The concentrations of cyanide lay in the same range as those in Table VIII. When the electrocardiograph registered the return of a normal rhythm, the amplitude of the beat was large in the first trial of expt. 59 and appreciable in expts. 57 and 58. When the perfusion fluid was changed to one which was cyanide-free, the amplitude in expt. 58 rose from 10 to 60 mm. In the second trial of expt. 58, and in expt. 60, cyanide did not arrest fibrillation.

In addition to the results given in Tables VIII and IX there are 10 other results in which the effect of cyanide could not be stated. Since cyanide may have two opposite effects it is possible that in these experiments the two effects in some way neutralized one another.

DISCUSSION

The observations that azide, fluoride and, perhaps less conclusively, monoiodoacetate increase the occurrence and duration of ventricular fibrillation are not surprising in view of the general belief that, when there is interference with metabolic processes, fibrillation is more likely to occur. Dinitrophenol has already been shown to have a similar effect (Armitage *et al.*, 1957), and this is possibly due to an uncoupling of the linkage between oxidation and phosphorylation, leading to diminished formation of adenosine triphosphate. To what are the actions of azide and fluoride due ? Since it seemed possible that in their presence less energy would be available from glycolysis, the effect of lack of glucose was investigated and it was found to exert a striking fibrillatory action. If a heart was perfused with a glucose-free solution sufficiently long, sometimes it fibrillated spontaneously, and it always fibrillated after electrical stimulation. This fibrillation was arrested by adding glucose, and the restorative power of glucose was assisted by insulin, which facilitates the entry of glucose into cells. In some experiments mannose, and in others pyruvate, had the restorative power of glucose. L(−)lactate however, had not.

Up to this point the findings seemed acceptable. An unexpected observation was then made on the effect of temperature. When hearts were caused to fibrillate at 37°, they ceased to do so at 32°. On raising the temperature to 37°, they fibrillated again. Beaulnes and Day (1957) had made a similar observation in isolated atria. It may, however, be that the effect of temperature in the isolated heart perfused with a modified Ringer solution is related to the supply of oxygen. This is the amount carried in solution, and is much smaller than is available when blood flows through the coronary vessels. It is not unlikely that the oxygen in Ringer solution is sufficient to maintain the metabolism at 32°, but not the increased metabolism at 37°. Thus stimulation at 32° is not followed by fibrillation because the oxygen supply is adequate ; when, however, the temperature is raised to 37° stimulation is followed by fibrillation because the oxygen supply is no longer adequate.

The fibrillatory action of adrenaline may have a similar basis. When adrenaline is perfused through the heart it greatly augments the rate and force of the beat; this must lead to a greater demand for oxygen, which perhaps cannot be met by the amount dissolved in the perfusion fluid. As a result there is an oxygen deficiency and a greater tendency to fibrillation. It is possible that the occurrence of ventricular fibrillation in the body as a result of the injection of adrenaline during chloroform anaesthesia has a similar explanation, if chloroform diminishes the availability of the energy supplied by oxidative processes.

The observations, however, do not give direct support to this view of the importance of the oxygen supply. Yet they may do so indirectly because of some of the results obtained with cyanide. In some experiments the presence of cyanide had a similar effect to the presence of azide or of fluoride. It increased fibrillation. This may indicate the effect of a partial reduction in oxidative processes.

Direct observations on a reduction of oxygen supply were, however, confined to a total exclusion of oxygen and to a reduction of oxygen to 10% in the aerating mixture. With a total exclusion of oxygen came the unexpected result that fibrillation was arrested. In a proportion of experiments with cyanide a similar result was obtained. Thus it appeared that when the cytochrome system was completely out of action fibrillation could not continue.

It is perhaps interesting to note that there are two other circumstances in which fibrillation cannot occur in the isolated heart; the one is in the absence of calcium and the other is in the presence of twice the normal concentration of potassium in the perfusion fluid.

Is any difference known between the isolated heart when fibrillating and when beating normally? Holland (1957) first demonstrated that, in the isolated atria, fibrillation as a result of a high rate of stimulation began when the net loss of K^+/unit of time exceeded a certain figure. Later Armitage *et al.* (1957) showed that when the ventricles of the isolated heart were fibrillating there was an increased loss of K^+ as compared with the periods of normal beating preceding and following. This observation may be related to the fact that fibrillation can be arrested by increasing the K^+ concentration in the perfusing fluid.

The results in the present work may possibly indicate that to avoid fibrillation it is necessary to facilitate the active transport of K^+ into the cell. The high net loss of K^+ in fibrillation may be due to insufficient active transport, and the defibrillatory effect of an increased external concentration of K^+ may be due to an improved active transport. This conception sheds light on the fibrillatory effect of a glucose-free medium, which may be due to lack of the substance whose breakdown within the cell normally provides the energy for the active transport of K^+. The fibrillatory effect of the metabolic inhibitors which have been studied may likewise be due to their inhibition of processes which release the energy required for active transport of K^+.

It is clear that the results obtained raise many queries. The removal of glucose by an isolated heart from the fluid perfusing it can be studied when the perfusion fluid is recirculated in a closed system (Burn and Dale, 1924) and it is desirable to know whether this removal is less in the presence of metabolic inhibitors and particularly of fluoride. It would then be possible to say whether the fibrillatory action of fluoride was due to inhibition of glycolysis or whether to some other action such as the binding of intracellular, but not enzymatic, magnesium. Much might also be learnt from observations of oxygen consumption. Perhaps the chief value of the results is that they point to the ways in which a study of the biochemical aspects of fibrillation can be pursued.

I would like to express my thanks to Professor J. H. Burn for stimulating guidance in this work, and to the Smith, Kline and French Laboratories for a maintenance grant. The electrocardiograph was very kindly supplied to the Department by the Wellcome Trust.

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COMPARISON OF THE EFFECTS OF ESERINE AND NEOSTIGMINE ON THE LEECH MUSCLE PREPARATION

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The relative potencies of eserine and neostigmine were determined on three preparations of cholinesterase. Eserine was found to be twice as potent as neostigmine on the pseudocholinesterase of horse plasma, half as potent on the true cholinesterase of cat central nervous system, but twelve times as potent on the true cholinesterase prepared from the leech body wall. On the leech preparation, about ten times smaller concentrations of eserine than of neostigmine were required to potentiate the acetylcholine response, but after removal of the anticholinesterase from the bath fluid, the potentiation persisted much longer after neostigmine than after eserine. The greater sensitivity of the leech muscle to eserine is fully accounted for by the fact that its cholinesterase is more sensitive to eserine than to neostigmine. The longer lasting potentiation after neostigmine on the other hand suggests that this anticholinesterase becomes more firmly attached to the cholinesterase receptors in this muscle than does eserine.

In experiments on perfused cerebral ventricles of the cat reported elsewhere (Bhattacharya and Feldberg, 1958), it became necessary to assay small amounts of eserine and neostigmine in samples of perfusate. When an attempt was made to assay these samples by their ability to sensitize the leech muscle preparation to acetylcholine, it became evident that the two anticholinesterases showed certain differences. These are reported in the present paper, which also includes a comparison of the potency of eserine and neostigmine in inhibiting preparations of pseudo- and true cholinesterase.

METHODS

Assay of Cholinesterase Inhibiting Activity.—As a source of pseudocholinesterase, samples of freeze-dried oxalated horse plasma were used. The samples had been stored in the cold for several years but had retained potent anticholinesterase activity. On the day of testing, a dried sample corresponding to 10 ml. of plasma was dissolved with frog Ringer solution so as to obtain a dilution of 10% plasma. Two preparations were used as source of true cholinesterase: either the caudate nucleus and thalamus of the cat or the dorsal body wall of the leech (*Hirudo medicinalis*). The caudate nucleus and the thalamus were removed from a freshly killed cat, weighed and homogenized with 5 ml. of distilled water/g. tissue in a small glass homogenizer The homogenate was kept in the deep freeze and was used for several days. On the day of testing, a sample of homogenate was diluted 1:50 with frog Ringer solution. The dorsal body wall of the leech was dissected free, cut into small pieces, freeze-dried and powdered. A solution of 3 mg./ml. in frog Ringer solution was prepared on the day of testing.

The method of assay was essentially that described by Burn (1952) in which a given dose of acetylcholine is incubated for a specified time with a given amount of cholinesterase and anticholinesterase and then assayed on the frog rectus muscle for acetylcholine. If the amounts of acetylcholine and cholinesterase are properly chosen the amount of acetylcholine remaining undestroyed in the sample after incubation depends on the amount of anticholinesterase added. In the present experiment, the incubation volume was 4 ml. and consisted of 1 ml. of either diluted plasma or diluted homogenate, 1 ml. acetylcholine 1:1,000,000 and 2 ml. of frog Ringer solution, alone or with varying amounts of anticholinesterase. The anticholinesterase was kept in contact with the cholinesterase for 15 min. before the acetylcholine solution was added. The mixture was incubated for 10 min. at room temperature. The enzyme activity was then stopped by the addition of 1 ml. eserine 1:40,000 so as to make the concentration of eserine in the now 5 ml. sample 1:200,000. The assay on the frog rectus muscle was made in a 5 ml. bath containing frog Ringer solution with eserine 1:200,000. Acetylcholine or the incubated samples were tested at 5 min. intervals and kept in the bath for 90 sec. With this procedure it was possible to assay a few ng. of eserine or neostigmine.

The Leech Muscle Preparation.—Two parallel strips from the dorsal body wall of the same leech

were suspended at room temperature, each in a separate 5 ml. bath. First the threshold concentration of acetylcholine was determined, which was about 1 : 500,000. This concentration was tested for 90 sec. at 20 min. intervals. When constant small contractions had been obtained, and 10 min. before the next dose of acetylcholine was given, eserine was added to the bath of the one and neostigmine to that of the other strip, the anticholinesterase being washed out immediately before.

Results

Inhibition of Cholinesterase.—Table I shows the amounts of eserine and neostigmine in μg. which gave the same degree of inhibition of cholinesterase by our method of testing. Neostigmine was half as potent as eserine when tested on pseudocholinesterase. The results obtained with true cholinesterase differed with the two preparations used. In the experiments with homogenate of cat central nervous system, neostigmine was found to be twice as potent as eserine, but in the experiments with powder prepared from the leech body wall, which were suggested to us by Sir Henry Dale, eserine was found to be twelve times as potent as neostigmine. This is illustrated in Fig. 1, which is part of an assay on a frog rectus muscle. The unlabelled contractions were caused by 1 μg. acetylcholine alone, the small contraction at C by 1 μg. acetylcholine after incubation with 3 mg./ml. of powder prepared from the leech body wall. This contraction thus shows the destruction of acetylcholine on incubation with this concentration of cholinesterase. The other contractions were obtained after incubation of 1 μg. acetylcholine with the same amount of cholinesterase, but after the addition of different amounts of eserine or neostigmine. It will be seen that with 0.1 μg. of neostigmine the inhibition of the cholinesterase was less, with 0.15 μg. more than with 0.01 μg. eserine, but that with 0.12 μg. neostigmine it was about equal.

Table I also includes a comparison with dyflos (DFP), a selective inhibitor of pseudocholinesterase. Compared with either eserine or neostigmine, it inhibited the pseudocholinesterase in smaller doses, and the true cholinesterase of the cat central nervous system in larger doses.

In Table II, these results are compared with those obtained by other authors using different methods. Dr. V. C. Abrahams kindly measured for us the cholinesterase-inhibiting properties of eserine and neostigmine with the Warburg technique. His results have been included in this Table, which gives the ratio of potency: eserine/neostigmine. In calculating this ratio, the molecular weights of the various salts used by different authors have been taken into account. The molecular weight of eserine is 275, of its hydrochloride 312, of its salicylate 414, and of its sulphate 649. But since the sulphate contains two moieties of eserine half the molecular weight, 324, was used. The molecular weight of neostigmine

Table I

THE WEIGHTS IN μG. OF ANTICHOLINESTERASE GIVING THE SAME DEGREE OF INHIBITION OF CHOLINESTERASE

The numerals in brackets are molecular weights except in the case of eserine sulphate, which contains two eserine moieties, half the molecular weight.

	True Cholinesterase		Pseudo-cholinesterase
	Cat C.N.S.	Leech	
Neostigmine methyl-sulphate (334) ..	0·04	0·12	0·04
Eserine sulphate (324)	0·08	0·01	0 02
Dyflos (185) ..	3·0		0·005

Fig. 1.—Contractions of the eserinized frog rectus muscle suspended in a 5-ml. bath. The unlabelled contractions are in response to 1 μg. acetylcholine; the contraction at (C) is to 1 μg. acetylcholine after 10 min. incubation in 5 ml. containing 15 mg. powder of leech body wall; those at (N) and (E) are to 1 μg. acetylcholine similarly incubated, but after the cholinesterase had been in contact for 15 min. with either neostigmine (N) or eserine (E), the numerals indicating in μg. the amounts of anticholinesterase used. In the assay, the contractions to the incubated samples were always followed by two contractions to 1 μg. acetylcholine, but only one of these is shown in the first part and none in the second part of the record.

is 223, of its bromide 303, and of its methylsulphate 334. Thus eserine sulphate and neostigmine methylsulphate, the salts used in the present experiments, can be compared without great error on a weight-for-weight basis.

Whereas Roepke (1937) found neostigmine 3 times, and Blaschko, Bülbring and Chou (1949) 1.5 times, as active as eserine in inhibiting pseudocholinesterase, Eadie (1942), Abrahams (personal communication) and ourselves found the reverse, that eserine was 1.6 to 2 times as active as neostigmine. On the other hand, on the true cholinesterase of the cat or dog central nervous system, we found eserine half as active as neostigmine; Hawkins and Mendel (1946) and Abrahams (personal communication) found both equally active, and Blaschko *et al.* (1949) found eserine 4 times as potent as neostigmine. On the true cholinesterase of the electric eel, Cohen and Unna (1951) found eserine 10 times as active as neostigmine. This corresponds to our results obtained on the true cholinesterase of the leech muscle; on this preparation eserine was 12 times as active as neostigmine.

Effect on Leech Muscle.—Neither eserine nor neostigmine caused contraction of the leech muscle when given at 10 times the concentration used for comparing their potentiating effect. It was found that 0.05 μg./ml. eserine sulphate caused either approximately the same degree of potentiation as 0.5 μg./ml. neostigmine methylsulphate, or an even more pronounced potentiation as shown in the experiment illustrated in Fig. 1. Eserine is thus at least 10 times as potent as neostigmine.

Bülbring (1946), Bülbring and Chou (1947) and Cohen and Unna (1951) have compared the effect of eserine and neostigmine on other striped muscle preparations. Their results, together with our own, are given in Table II. In potentiating the frog rectus muscle to acetylcholine, eserine was found to be only 1.8 times as potent as neostigmine and the anticurare action of eserine on the rat diaphragm was only 1.2 times greater than that of neostigmine. On the other hand, in potentiating the muscle twitch of the rat diaphragm, eserine was found to be active at about 1/10 of the concentration of neostigmine. This difference corresponds to that found for the potentiation of acetylcholine on the leech muscle. In our experiments an additional difference in the action of eserine and neostigmine on the leech muscle was observed. It concerned the duration of potentiation after washing out the anticholinesterase. After eserine, it took the muscle a shorter time to revert to its original sensitivity to acetylcholine than after neostigmine (Fig. 2).

In a few experiments of this kind the effect of dyflos caused about the same degree of potentiation as 0.5 μg./ml. of neostigmine or 0.05 μg./ml. of eserine, but after dyflos it took an even longer time for the muscle to revert to its original sensitivity to acetylcholine than after neostigmine.

TABLE II

COMPARISON OF POTENCY OF ESERINE AND NEOSTIGMINE ON CHOLINESTERASE AND STRIPED MUSCLE PREPARATIONS

Authors	Ratio of Activity Eserine/ Neostigmine	Pseudo-cholinesterase	True Cholinesterase	Method	Effect on Striped Muscle Preparations
Roepke (1937)	0·33	Horse serum		Titration	
Eadie (1942)	1·6	Dog ,,		Electrometric titration	
Blaschko, Bülbring, and Chou (1949)	0·67	Horse ,,		Warburg 50% inhibition	
Abrahams (personal communication)	1·6	Horse plasma (dried)		Warburg inhibition at equal concentration	
Bhattacharya and Feldberg (1958)	2	Horse plasma (dried)		Frog rectus assay	
Hawkins and Mendel (1946)	1		Dog caudate nucleus	Warburg 50% inhibition	
Blaschko, Bülbring, and Chou (1949)	4		Dog caudate nucleus	,, ,, ,,	
Cohen and Unna (1951) ..	10		Electric eel	,, ,, ,,	
Abrahams (personal communication)	1		Cat caudate nucleus	Warburg inhibition at equal concentration	
Bhattacharya and Feldberg	0 5		Cat caudate nucleus	Frog rectus assay	
,,	12		Leech muscle	,, ,, ,,	
Bülbring (1946) ,, .. ,, ..	10				Augmentation of muscle twitch (rat diaphragm)
Bülbring and Chou (1947) ..	1·2				Anticurare activity (rat diaphragm)
Cohen and Unna (1951) ..	1·8				Acetylcholine potentiation of frog rectus muscle
Bhattacharya and Feldberg	10				Acetylcholine potentiation of leech muscle

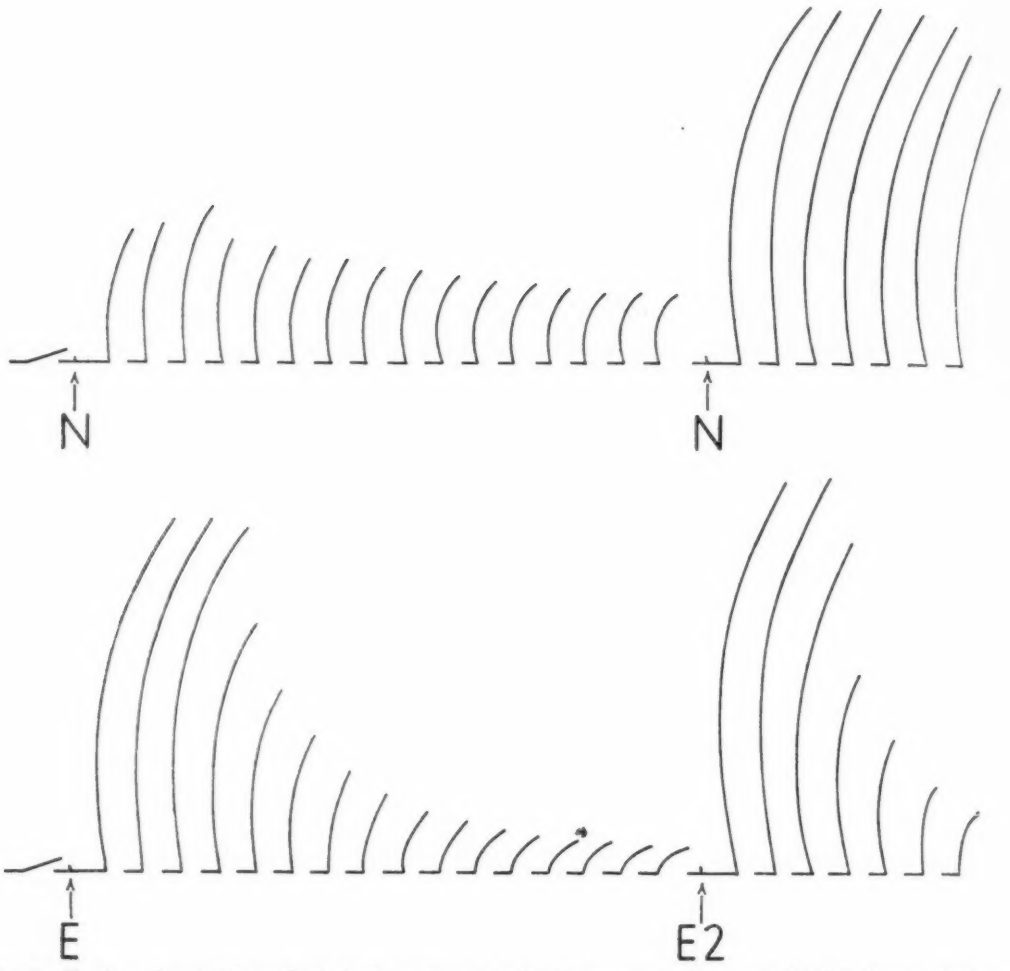

FIG. 2.—Tracings of contractions of twin leech muscle preparations in response to 10 μg. acetylcholine given at 20 min. intervals and kept in the 5-ml. bath for 90 sec. At N, E, and E2 the bath fluid contained for 10 min. before the subsequent acetylcholine contractions: 2.5 μg. neostigmine (N), 0.25 μg. eserine (E), and 1·25 μg. eserine (E2). The anticholinesterases were washed out immediately before the next acetylcholine contraction.

DISCUSSION

Eserine and neostigmine are generally looked upon as being anticholinesterases of about equal potency. However, in those experiments in which actual comparisons have been made, divergent results have been obtained. Some authors found eserine, others neostigmine, more potent in inhibiting pseudocholinesterase; in our experiments eserine was about twice as potent as neostigmine.

When the drugs were tested as inhibitors of true cholinesterase some authors found no difference between the two, others found eserine more active than neostigmine. We found a difference according to the source of the true cholinesterase. On homogenate of cat caudate nucleus and thalamus, eserine was only half as active as neostigmine, but on the true cholinesterase prepared from the leech body wall eserine was 12 times as active as neostigmine. The finding that eserine potentiates the acetylcholine contraction of the leech muscle in about 1/10 of the dose of neostigmine is thus fully accounted for by the greater sensitivity of the cholinesterase of this muscle to eserine. It would be interesting to know whether the cholinesterase of the rat diaphragm is also more sensitive to eserine than to neostigmine, because Bülbring (1946) found that eserine augmented the muscle twitch on this preparation in 1/10 of the dose of neostigmine.

The fact that the relative potencies of eserine and neostigmine vary so much on different preparations of true cholinesterase has to be taken into

account when trying to interpret in terms of anticholinesterase activity responses obtained on whole organs with these anticholinesterases. In addition, other factors, such as penetration into the tissue, a firmer attachment to the enzyme receptors may influence the response. For instance, the finding that the effect of neostigmine in potentiating the leech muscle to acetylcholine persisted longer than the potentiating effect of eserine after the anticholinesterase had been washed out may indicate that neostigmine becomes more firmly attached to the enzyme receptors and is thus a less reversible inhibitor than eserine. Firmer attachment may also occur when neostigmine is brought into contact with cholinesterase preparations *in vitro*, but it need not necessarily be revealed in measuring the inhibition by the usual methods. On the other hand, it may be a feature characteristic for the organization of the enzyme in the structure of the muscle fibre.

If the longer duration of the potentiating effect of neostigmine on the leech muscle, which is suggestive of a firmer attachment to the receptors of the muscle cholinesterase, were to apply to striated muscles and motor endplates in general, and particularly to those of human beings, this might explain why neostigmine is more effective in myasthenia gravis than eserine.

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PERFUSION OF CEREBRAL VENTRICLES: EFFECTS OF DRUGS ON OUTFLOW FROM THE CISTERNA AND THE AQUEDUCT

BY

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In cats under chloralose anaesthesia the cerebral ventricles were perfused with Locke solution at a rate of 0.1 ml./min. from an indwelling cannula in the lateral ventricle. The effluent was collected from a cannula either inserted into the cisterna magna or pushed into the aqueduct. When collection was from the cisterna the perfusion included relatively large areas of the subarachnoidal spaces since in cats the foramina of Luschka form the only outlet from the fourth ventricle. Tubocurarine, histamine, and adrenaline injected intravenously caused great variations in outflow from the cisterna, but these changes did not occur when the collection was from the aqueduct. The changes in outflow from the cisterna were similar whether the injection produced a fall of arterial blood pressure as after tubocurarine and histamine, or a rise, as after adrenaline.

In the past, increased formation of cerebrospinal fluid (c.s.f.) by drug action was sometimes based on experiments in which an increased rate of leakage of c.s.f. from a cannula in the cisterna magna had occurred (Dixon and Halliburton, 1913; Weed and Cushing, 1915). As Davson (1956) points out, such an increased leakage does not necessarily signify increased secretion, "... for example, the normal drainage routes could be hindered by the effects of the drugs on the vascular pressures, and so on." In the present experiments the changes in flow of c.s.f. produced by drug action were observed by procedures which permitted differentiation between such effects and secretion from the choroid plexuses.

The methods used consisted of perfusion of the cerebral ventricular system in anaesthetized cats in such a way that the effluent could be collected from either the cisterna magna or the aqueduct of Sylvius. When the outflow was from the cisterna the perfusion fluid was in communication with the whole subarachnoid space, whereas when the outflow was from the aqueduct, perfusion was confined to the lateral and third ventricles. Changes in flow resulting from changes in secretion of the ventricular choroid plexuses are evident with both methods. Changes due to extrachoroidal c.s.f. formation, or passive adaptation in response to changes in the perivascular spaces of the brain, either do not occur, or occur to a slight degree only, when perfusion is confined to the lateral and third ventricles.

The present experiments deal with the effects of intravenous injections of tubocurarine, histamine and adrenaline, using both methods of collection.

The experiments were the outcome of work reported elsewhere (Bhattacharya and Feldberg, 1957, 1958) on the release of acetylcholine in the effluent from the perfused ventricles and on the appearance in this effluent of anticholinesterases injected intravenously. In these experiments it became necessary to inject tubocurarine intravenously in order to abolish muscular contractions, and it was found that these injections affected the outflow from the perfused ventricular system. Since tubocurarine releases histamine, and histamine releases adrenaline and noradrenaline from the suprarenals, the question arose as to whether the changes in outflow produced by tubocurarine were brought about indirectly by the release of these substances.

METHODS

The experiments were carried out in cats under chloralose anaesthesia. The cerebral ventricular spaces were perfused with Locke solution at a constant rate of inflow of 0.1 ml./min. from the cannulated left lateral ventricle. Fig. 1 is a diagrammatic representation of the two methods of collecting the outflow. The method of collection from the

cisterna magna, as illustrated in Fig. 1 (*a*), was the same as that described previously (Bhattacharya and Feldberg, 1957). In cats there is no foramen of Magendie, therefore the perfusion fluid passes from the foramina of Luschka at the lateral recesses of the fourth ventricle through a relatively large region of the subarachnoidal space before it reaches the cannula in the cisterna. This was shown when at the end of such a perfusion experiment methylene blue was injected through the ventricular cannula. It deeply stained the ventral part of the floor of the fourth ventricle, but not the caudal part beyond the lateral recesses ; however, the outer surface of the brain stem was deeply stained and the stain extended to the dorsal surface of the colliculi and to the ventral surface of the occipital pole of the cerebrum.

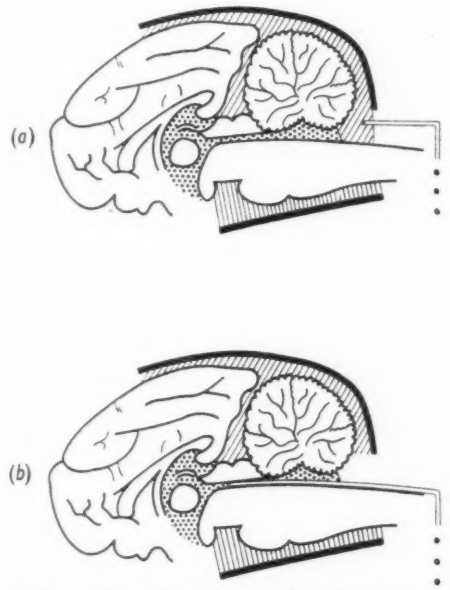

FIG. 1.—Diagrams of a median sagittal section of the brain of a cat, illustrating the two methods of collection of outflow during perfusion from the cannulated lateral ventricle. (*a*) Outflow from cisterna magna, (*b*) from the cerebral aqueduct. Dotted areas, ventricular spaces; shaded area, subarachnoidal spaces. The lateral ventricle is not shown. For details see text.

On the other hand, it is clear from diagram in Fig. 1 (*b*) that, when the cannula is in the middle of the aqueduct and fully fills its lumen, the perfusion is confined to the lateral and third ventricles and to the beginning of the aqueduct. The following procedure was adopted in order to insert the cannula into the aqueduct. The layers of muscle covering the atlanto-occipital membrane were dissected away and the membrane cut so as to expose the medulla. The lower part of the cerebellum was exposed by nibbling away the margins of the supra-occipital bone at the border of the foramen magnum. This made it possible to lift the cerebellum gently with a small curved spatula from the medulla and to insert a fine polythene tube along the floor of the fourth ventricle into the aqueduct. The outside diameter of this tube was 2 mm. and at its tip it was narrowed to 1 mm. The distance from the margin of the cerebellum to the middle of the aqueduct, where anterior and posterior colliculi meet, is 2 cm. or a little more. The tube was therefore inserted for at least 2 cm. beyond the margin of the cerebellum and the position of the tip of the cannula in the aqueduct was ascertained at the end of the experiments as follows. Either perfusion was stopped and 1 ml. of 1% methylene blue solution was slowly injected with a syringe through the intraventricular cannula, or the ventricular spaces were perfused for a few minutes with Locke solution containing methylene blue before the cat was killed. If the cannula was in the right position, the dye flowed out only through the polythene tube. If traces of dye seeped out around the tube, it meant that the tip of the cannula had not reached the aqueduct and that at least part of the fourth ventricle was not excluded from the perfusion. In this case the experiment was discarded. A further check was made after killing the cat by dissecting the brain without removing the tube and locating its tip in the aqueduct. With the cannula in the right position there was a sharp demarcation in the middle of the aqueduct between the heavily stained and the unstained tissue indicating the end of the perfused area.

To measure the outflow each drop was signalled on the smoked drum, or the intervals in seconds between each drop were noted. The outflow was collected in 10 or 20 min. samples, the total volumes of which were measured, and as the number of drops for each sample was counted, it was easy to convert the drops into fractions of a ml.

For intravenous injections the left femoral vein was cannulated. In some experiments, in which records of the arterial blood pressure were taken, the femoral artery of the other side was cannulated and connected with a mercury manometer. As anticoagulant, 0.5 to 1 ml. of 1% solution of heparin was injected into the arterial cannula. In those experiments in which tubocurarine was given, the cat was artificially ventilated through the cannulated trachea. For the injection, tubocurarine chloride, histamine acid phosphate and adrenaline chloride were used. All weights of histamine refer to the base, those of tubocurarine and adrenaline to the salt. The intravenous injections were made in 1 ml. solution washed in by 2 ml. saline solution.

RESULTS

When the cisterna had been cannulated, the spontaneous outflow of c.s.f. practically ceased when about 1 ml. had been collected ; perfusion with Locke solution was then started and the outflow collected in 20 min. samples. The rate of outflow, which at first was greater than the rate of inflow, gradually approximated to the rate of inflow. This is illustrated by the experiments in Table I, in which the outflow during the first

TABLE I

VOLUME OF OUTFLOW FROM THE CISTERNA DURING PERFUSION WITH LOCKE SOLUTION IN SUCCESSIVE 20 MIN. SAMPLES

Expt. No. 1	Expt. No. 2	Expt. No. 3
2·35	2·9	2·45
2·25	2·4	2·05
2·05	2·4	2·1
1·95	2·3	2·1
2·15	2·2	2·1
1·95	2·2	
	2·05	
	2·05	

20 min. sample exceeded the inflow by 0.35 to 0.9 ml. but slowed down in the following samples. In experiment No. 3, it approximated to the inflow during the second sample; in Experiment No. 2, only after 2 hr. of perfusion. The outflow showed irregular large fluctuations which were not dependent on respiration or on changes in arterial blood pressure. The experiments of Figs. 2 and 3 illustrate these fluctuations by differences of the interval in seconds between each drop. These fluctuations were not abolished when artificial ventilation was begun; the outflow then slowed down for a few minutes.

When the outflow was collected from the aqueduct, it was either equal to the inflow, or exceeded it by 0.05 to 0.15 ml. in 20 min., even under those conditions in which the cisternal outflow would have been much greater. The outflow from the aqueduct showed irregular fluctuations (Figs. 2 and 3), but they were often less pronounced than the fluctuations seen in the cisternal outflow.

In the experiments in which arterial blood pressure was recorded, 0.5 to 1 ml. of 1% heparin solution was injected into the blood pressure cannula. In several of these experiments the outflow became blood-stained after a time. Further, the injection of tubocurarine, histamine, and particularly of adrenaline, frequently led to the appearance of blood in the outflow. After a time the blood gradually disappeared from the effluent, only to reappear with a renewed injection. In some experiments the contamination with blood became so extensive as to give the impression that the outflow consisted mainly of blood; in this event the experiment had to be terminated. The contamination of the effluent with blood occurred whether the outflow was from the cisterna or from the aqueduct. It was found, post mortem, that the cannulated left lateral ventricle contained varied amounts of blood. The appearance of blood in the effluent was therefore attributed to bleeding from the injured tissue of the needle tract in the brain substance as a result of the heparin entering the circulation, and accelerated either by vasodilatation around the injured region produced by tubocurarine or histamine, or by the strong pressor action of adrenaline. The effects on the outflow described in the following paragraphs, however, were independent of this complication and the illustrations are from experiments in which the outflow was not contaminated with blood.

Tubocurarine.—The changes in outflow from the cisterna after an intravenous injection of 1 mg. of tubocurarine varied somewhat from experiment to experiment and also with repeated injections. Usually, the injection caused first an acceleration in outflow, which after the first minute was interrupted by stoppage of the flow for a minute or even longer. During this time the flow not only stopped but the fluid was drawn back into the cannula, and a drop which had formed and was about to fall was seen to be drawn into the cannula. When the flow recommenced it soon became 2 or 3 times as fast as before the injection. This is illustrated in the experiment shown in Fig. 2, in which the time for the formation of each drop is plotted in seconds as ordinate, each vertical line representing one drop. The horizontal broken line indicates the rate of inflow; with the

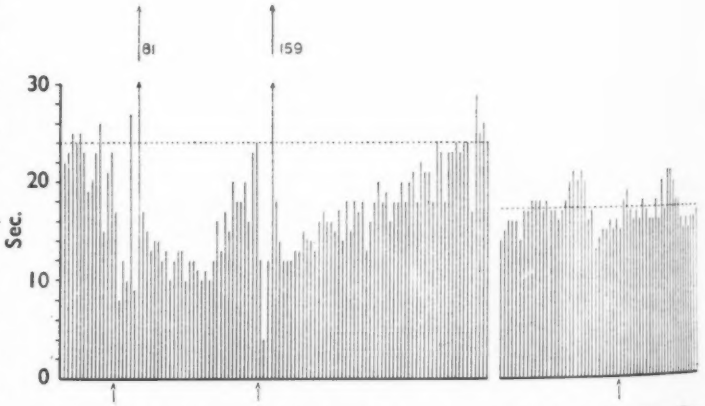

FIG. 2.—Effect of intravenous injections of 1 mg. tubocurarine (at arrows) on outflow from perfused cerebral ventricles of a cat under chloralose anaesthesia. Collection from cisterna on the left and from aqueduct on the right. Each vertical line represents one drop. Ordinate, interval between drops in sec. The numbers 81 and 159 refer to interval in sec. Horizontal broken line, rate of inflow (2 ml./20 min.). The diameter of the cannula used for collection from the aqueduct was smaller than from the cisterna, so that the volume of drops decreased and the time interval between drops was shortened. (For details see text.)

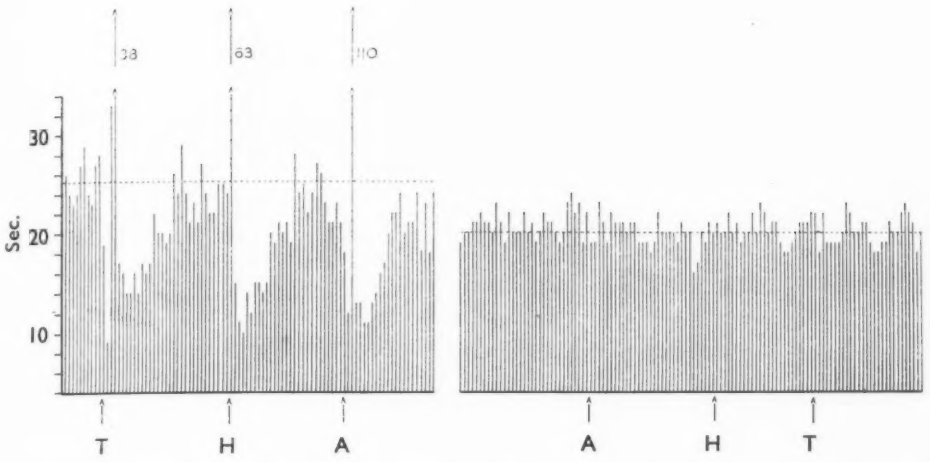

FIG. 3.—Effect of intravenous injection of 1 mg. tubocurarine (T), 10 μg. histamine (H) and 25 μg. adrenaline (A) on outflow from perfused cerebral ventricles of a cat under chloralose anaesthesia. Collection from cisterna on the left and from aqueduct on the right. Each vertical line represents one drop. Ordinate interval between drops in sec. The numbers 88, 63, and 110 refer to intervals in sec. Horizontal broken line, rate of inflow (2 ml./20 min.). The diameter of the cannula used for collection from the aqueduct was smaller than from the cisterna, so that the volume of drops decreased and the time interval between drops was shortened. (For details see text.)

cannula used it was equivalent to an outflow of one drop every 24 sec. Before the injection the outflow was a little in excess of the inflow as shown by the fact that most of the intervals were less than 24 sec.; during the 10 min. preceding the injection 1.1 ml. was collected. The two injections of 1 mg. tubocurarine which followed each other at 10 min. intervals produced each time an acceleration of outflow for 3 to 4 drops followed by a slowing down or stoppage of the outflow, so that the interval for one drop became 81 sec. after the first and 159 sec. after the second injection of tubocurarine. The outflow then quickly increased, remained high for a few minutes and then returned gradually to the pre-injection rate of flow. The overall effect was an increased outflow which amounted to 1.4 ml. in 10 min. after the first and to 1.3 ml. in 10 min. after the second injection; it then became practically normal again and amounted to 1.15 ml. in the next 10 min. sample. This experiment thus illustrates that intravenous tubocurarine can cause an increased outflow from the cisterna. In other experiments in which the injection of tubocurarine produced similar variations in outflow from the cisterna, the overall effect was small and sometimes even absent. For instance, in the experiment shown in Fig. 3 the intravenous injection of 1 mg. tubocurarine caused the same variation, acceleration, slowing down and acceleration of outflow; the overall effect showed scarcely any increase in outflow because 1.1 ml. was collected in the first 10 and 1.7 ml. in the first 15 min. after the injection, as compared with 1.05 to 1.1 ml. in preceding 10 min. samples.

When collection was from the aqueduct the intravenous injection of 1 mg. tubocurarine produced practically no change in outflow, whether the tubocurarine had increased the overall outflow from the cisterna or not (Figs. 2 and 3).

Fig. 4 (*a*) and (*b*) illustrates two experiments in which the blood pressure from the femoral artery was recorded. The initial acceleration of outflow from the cisterna which followed the intravenous injection of 1 mg. of tubocurarine occurred before the main depressor effect. This depressor effect was preceded by a small pressor effect. A similar but even smaller rise of blood pressure was produced by an intravenous injection of saline solution. The initial acceleration in outflow from the cisterna coincides with this rise in blood pressure. When the outflow begins to slow down or to stop, the blood pressure may still be elevated or it may already have begun to fall. If the slowing is only transient as in experiment Fig. 4(*a*), the subsequent acceleration begins whilst the pressure is at its lowest level. In experiment Fig. 4(*b*), on the other hand, the stoppage of outflow lasted longer and the subsequent acceleration of outflow began after the blood pressure had practically recovered and a secondary more sustained fall had occurred. When the injections of tubocurarine were repeated at 10 or 20 min. intervals, it was often observed that after a few injections the effects on the out-

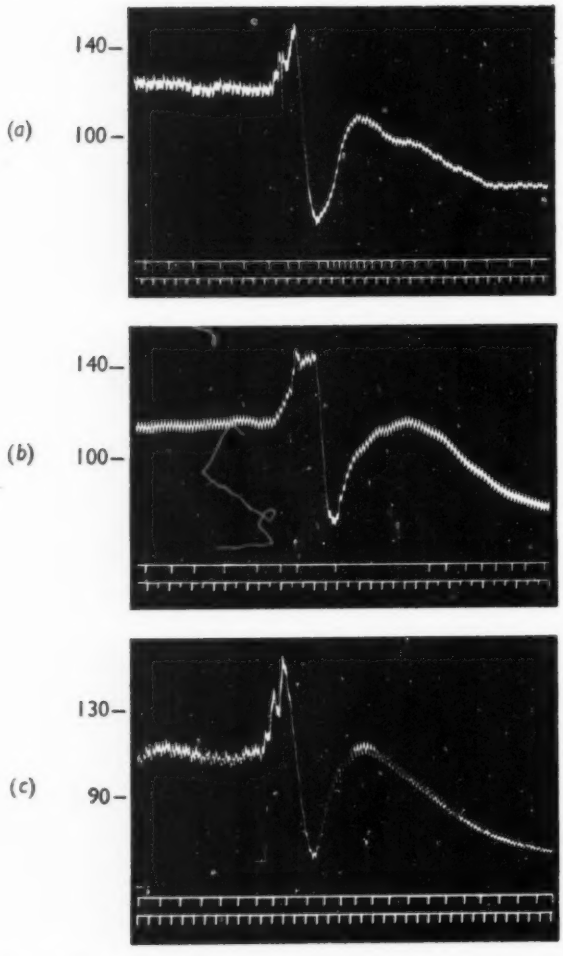

FIG. 4.—Effect of intravenous injections of 1 mg. of tubocurarine on arterial blood pressure and outflow from the perfused cerebral ventricles of three cats under chloralose anaesthesia. Each signal in the middle trace represents one drop. In cats (*a*) and (*b*) outflow was collected from the cisterna, in cat (*c*) from the aqueduct. Time (lowest trace), 10 sec.

flow diminished and were sometimes very small. When this happened the tubocurarine injections produced much smaller depressor effects, probably as the result of the diminished histamine release with the repeated injections.

Fig. 4(*c*) shows that, when collection was from the aqueduct, the intravenous injection of tubocurarine no longer affected the outflow although it caused pronounced changes in blood pressure. In this experiment there was a slight acceleration of outflow which was not regularly observed and was within the limit of the normal fluctuations.

Histamine.—The changes in outflow from the cisterna after an intravenous injection of 10 μg. of histamine varied somewhat from experiment to experiment, but resembled those described for tubocurarine. In the experiment shown in Fig. 3 the injection caused a stoppage of outflow followed by pronounced acceleration, but there was scarcely any overall increase. In other experiments the changes in outflow consisted of acceleration followed by retardation, or of an initial short retardation followed by acceleration which in its turn was followed by retardation. These results are illustrated in Figs. 5 and 6 in which the arterial blood pressure was also recorded. In both experiments the increased outflow started after the blood pressure had fallen and continued during its recovery. When this acceleration was preceded by some retardation in outflow as in Fig. 5, the retardation occurred whilst the pressure was falling. The late reduction in outflow seen in Fig. 6 occurred after the blood pressure had risen. In some experiments the overall effect resulted in an increased outflow of about 0.1 ml. during the 10 min. following the injection ; in others, there was no overall increased outflow.

Figs. 3 and 5(*b*) show that histamine did not cause these changes in outflow when collection was from the aqueduct.

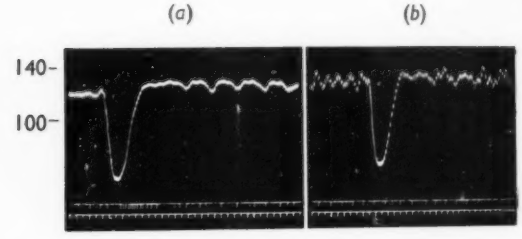

FIG. 5.—Effect of intravenous injections of 10 μg. of histamine on arterial blood pressure and outflow from the perfused cerebral ventricles of a cat under chloralose anaesthesia. At (*a*) outflow collected from cisterna, at (*b*) from aqueduct. Each signal in the middle trace represents one drop. Time, 10 sec.

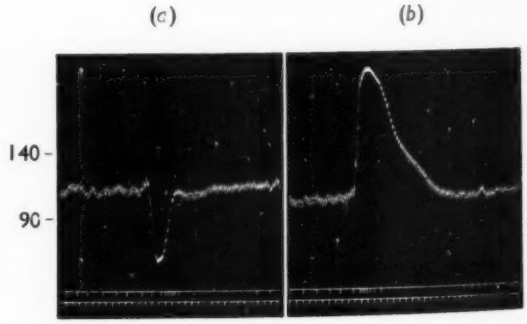

FIG. 6.—Effect of intravenous injection of 10 μg. of histamine (in *a*) and of 25 μg. of adrenaline (in *b*) on arterial blood pressure and outflow from the cisterna during perfusion of the cerebral ventricles of a cat under chloralose anaesthesia. Each signal in the middle trace represents one drop. Time, 10 sec.

Adrenaline.—Although intravenous injection of adrenaline caused a rise in blood pressure, the changes in outflow from the cisterna resembled those seen after histamine or tubocurarine (Figs. 3 and 6). In Fig. 3 the intravenous injection of 25 μg. adrenaline caused changes in outflow similar to those caused by a previous injection of 1 mg. tubocurarine. There was initial acceleration and then stoppage of the flow for nearly 2 min. followed again by a period of acceleration. In the experiment of Fig. 6 the arterial blood pressure was recorded also. The changes in outflow from the cisterna produced by adrenaline were similar to those produced by histamine although histamine caused a fall and adrenaline a rise in pressure. The acceleration of outflow which followed the injection of adrenaline coincided with the rise of pressure ; the stoppage of flow occurred when the pressure was falling again. Adrenaline always caused a prolonged stoppage of outflow whereas with tubocurarine and histamine the stoppage was sometimes transient. In some experiments with adrenaline the stoppage of flow was followed by some slight but definite acceleration as in Fig. 6.

These changes produced by adrenaline in the outflow from the cisterna caused either no overall effect in outflow or a slight increase.

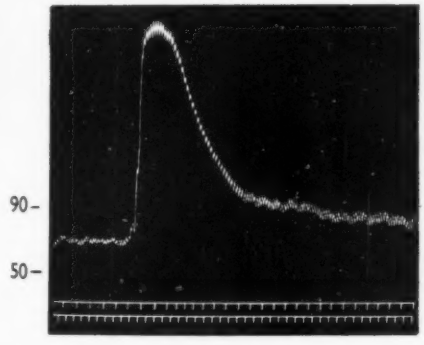

FIG. 7.—Effect of intravenous injection of 25 μg. of adrenaline on arterial blood pressure and outflow from the aqueduct during perfusion of the cerebral ventricles of a cat under chloralose anaesthesia. Each signal in the middle trace represents one drop. Time, 10 sec.

When the cannula was in the aqueduct the intravenous injection of 25 μg. of adrenaline did not produce the characteristic changes in outflow observed during collection from the cisterna. In some experiments, as in that shown in Fig. 7, there was a slight and transient acceleration of outflow during the rise in blood pressure ; in other experiments, the injections had no effect on the outflow.

DISCUSSION

The results of the present experiments on the perfused cerebral ventricles of the cat show that variations in outflow from the cisterna magna produced by intravenous injections of tubocurarine, histamine, and adrenaline cannot be attributed to secretion of c.s.f. from the choroid plexus of the lateral and third ventricles because the variations do not occur when the outflow is collected from the aqueduct. They must therefore be attributed to changes which occur either in the fourth ventricle or in the subarachnoidal space. As it is unlikely that the choroid plexuses of the fourth ventricle react to drugs differently from the choroid plexus of the third and lateral ventricles, the observed variations in cisternal outflow appear to be dependent on the inclusion of the subarachnoidal space in the perfusion system.

In the analysis of variations in cisternal outflow, a distinction must be made between variations in the production of c.s.f. and variations in the capacity of the subarachnoidal space resulting from blood pressure changes or from effects on the vessels covering the meninges. To a large extent the observed variations in cisternal outflow reflect adaptations in response to changes in the perivascular spaces of the central nervous system. This conclusion is based on the observation that acceleration was often followed by slowing or stoppage of the outflow and *vice versa,* so that the changes were to a great extent compensated. Further, when stoppage of outflow occurred, a drop which was partly formed was often seen to be drawn back into the cannula ; this indicated a transient increase of the available subarachnoidal space.

In some experiments adrenaline, which caused a rise in blood pressure, and histamine, which caused a fall, both produced an acceleration followed by slowing of the cisternal outflow. The acceleration after adrenaline could be explained by reduction of the subarachnoidal space brought about by an increase in the volume of the vascular bed in the meninges, due either to passive dilatation to the rise in arterial blood pressure, or to a dilator effect of adrenaline on the meningeal vessels. The subsequent slowing or stoppage of outflow which occurred when the blood pressure fell again could be the result of the re-establishment of the original capacity of the subarachnoidal space. On the other hand, the acceleration of outflow which occurred in the same animal after an intravenous injection of histamine could be the result of a vasodilator action of the histamine on the meningeal vessels. As long as the blood pressure

was low the reduction in subarachnoidal space due to this vasodilatation was compensated by the fall in arterial blood pressure. The acceleration in outflow commenced only when the blood pressure recovered, and the subsequent reduction or stoppage in outflow may represent the disappearance of the histamine vasodilatation in the meninges. The finding that blood pressure changes as well as vascular changes in the meninges affect the outflow, and sometimes in the opposite direction, would account for the fact that in different experiments the drugs affected the cisternal outflow somewhat differently.

Although the variations in cisternal outflow produced by the intravenous injections of drugs were to a large extent accounted for by changes in the relative capacity of the subarachnoidal space, there was also evidence for an increased production of c.s.f. In several experiments the volume of fluid collected from the cisterna during a 10 or 20 min. period after an intravenous injection of tubocurarine was greater than the inflow and greater than the volume collected during a similar period before the injections. This also happened to a lesser degree after an intravenous injection of histamine or adrenaline, and the only explanation would be that the production of c.s.f. had increased. Since the increased outflow did not occur under otherwise similar conditions when collection was from the aqueduct, it cannot be attributed to increased secretion from the choroid plexus of the lateral and third ventricles, and it is unlikely that the choroid plexus of the fourth ventricle would react differently from those of the lateral and third ventricles. The increased c.s.f. production is probably non-choroidal in origin, taking place in the subarachnoidal space. It may enter the subarachnoidal space by diffusion either from the brain substance or from the vessels of the meninges. This recalls observations of MacIntosh and Oborin (1953), who found that fluid transudes from the cat brain to a saline pool created at the surface of the cerebral cortex.

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PERFUSION OF CEREBRAL VENTRICLES: ASSAY OF PHARMACOLOGICALLY ACTIVE SUBSTANCES IN THE EFFLUENT FROM THE CISTERNA AND THE AQUEDUCT

BY

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In cats under chloralose anaesthesia the cerebral ventricles were perfused from a cannula in the lateral ventricle. The effluent was collected either from the cisterna or from the aqueduct. When acetylcholine was added to the perfusion fluid and its destruction was prevented by the addition of an anticholinesterase, its recovery in the internal effluent was incomplete and irregular. This could be explained mainly by the devious route the perfusion fluid had to take in order to reach the cisterna and the unavoidable mixture with the cerebrospinal fluid of the subarachnoidal space. When collection was from the aqueduct 90% or more of the acetylcholine added to the perfusion fluid was recovered. In the presence of an anticholinesterase in the perfusion fluid, acetylcholine appeared in the effluent. Neostigmine was more effective than eserine or dyflos. The acetylcholine originated mainly from structures lining the lateral and third ventricle because the amounts in the effluent from the aqueduct were only a little less than those in the cisternal effluent. When injected intravenously, eserine was found to pass more readily into the perfusion fluid than neostigmine, probably because eserine passes the blood-brain barrier more readily than neostigmine. The method provides a quantitative approach for the study of the blood-brain barrier for pharmacologically active substances.

Experiments in which the cerebral ventricles of the anaesthetized cat were perfused from the lateral ventricles and the outflow was collected either from the cannulated cisterna magna or from the cannulated aqueduct were described by Bhattacharya and Feldberg (1958b). Perfusate collected from the cisterna had traversed the lateral, third and fourth ventricles and part of the subarachnoidal space; perfusate collected from the aqueduct had passed through the lateral and third ventricles only. Drugs injected intravenously affected the outflow from the two points of collection in different ways. In the present experiments the two methods of perfusion have been used to examine the following problems. (1) What happens to substances added to the perfusion fluid? Can they be fully recovered in the effluent? The two substances examined were acetylcholine and histamine. (2) Does acetylcholine appear in the effluent when the ventricles are perfused with Locke solution containing an anticholinesterase or when an anticholinesterase is injected into the blood stream? (3) Do anticholinesterases injected into the blood stream pass into the perfusion fluid?

METHODS

The experiments were performed on cats anaesthetized intravenously with chloralose. The method of perfusion of the cerebral ventricles and the collection of the effluent from the cisterna or aqueduct was that described by Bhattacharya and Feldberg (1958b).

In experiments in which acetylcholine or histamine was added to the perfusion fluid in order to determine their recovery in the effluent, perfusion with the substances was allowed to proceed for about 20 min. before the first sample for assay was collected, in order to wash out the fluid present in the ventricular space. The effluent was then collected in successive 20 min. samples which were assayed either for acetylcholine on the frog rectus muscle or for histamine on the atropinized guinea-pig ileum.

In those experiments in which the appearance of endogenous acetylcholine in the effluent was examined, an anticholinesterase (eserine, neostigmine or dyflos) was injected into the circulation or added

to the perfusion fluid and the effluent was collected for successive 20 min. periods. These were assayed for acetylcholine on the eserinized leech muscle preparation. When the anticholinesterases were injected into the circulation by the intravenous (eserine or neostigmine) or by the intracarotid (dyflos) routes, it was necessary to counteract the peripheral actions by atropine and tubocurarine. Intravenous injections of eserine or neostigmine were given every 20 min., together with atropine and tubocurarine; the first dose was 1 mg. eserine or 1 mg. neostigmine together with 0.5 mg. atropine and 0.5 mg. tubocurarine and subsequent doses 0.25 mg. of each. In experiments with intracarotid injections of dyflos, 1 mg./kg. was injected into each carotid before the collection of the first sample was begun. As soon as there were signs of salivation and muscle twitching, 0.5 mg. of atropine and 0.5 mg. of tubocurarine were injected intravenously and sometimes again after 1 or 2 hr. In the experiments in which the anticholinesterases were added to the perfusion fluid it was necessary to inject atropine and tubocurarine only when concentrations as high as 1:50,000 were used. In such experiments, particularly with neostigmine and dyflos, salivation and frequent muscular twitching occurred during prolonged perfusion. When this happened 0.5 mg. of both atropine and tubocurarine were injected intravenously. In experiments in which the anticholinesterases were added to the perfusion fluid and injected intravenously as well, an initial period of perfusion varying from 1 to 4 hr. usually preceded the intravenous injections. The amounts of eserine or neostigmine injected intravenously were 1 mg. for the first and 0.25 mg. for all subsequent injections given at 20 min. intervals. At the same time atropine and tubocurarine were injected, the procedure being the same as described for the experiments with intravenous injections of eserine or neostigmine alone.

In experiments in which the passage of anticholinesterases from the blood stream into the perfusion fluid was measured, plain Locke solution was used for perfusion, and eserine, neostigmine or dyflos were injected intravenously. In some experiments with dyflos the injections were made into the carotid arteries. Intravenous injections were given when perfusion had gone on for 20 to 30 min. and before the first sample of effluent was collected. The anticholinesterase was injected together with tubocurarine and atropine; injections were repeated at 20 min. intervals. The amounts of anticholinesterase for the first injections were for eserine and neostigmine 1 mg. and for dyflos 1.75 mg., and a quarter of these amounts for all subsequent injections. The amounts of tubocurarine and atropine were 0.5 mg. for the first and 0.25 mg. for all subsequent injections. When dyflos was given into the carotid arteries, injections of the same amount were made once in each carotid. The effluent was collected for consecutive 40 min. periods and samples were assayed for their content of anticholinesterase as described by Bhattacharya and Feldberg (1958a).

In all experiments in which tubocurarine was injected intravenously artificial ventilation was applied through the cannulated trachea.

Atropine was used as sulphate, tubocurarine as hydrochloride, eserine as sulphate, neostigmine as methylsulphate, and acetylcholine as chloride. All values refer to the salts. Histamine was used as acid phosphate, and the values refer to the base.

RESULTS

Recovery of Histamine or Acetylcholine Added to the Perfusion Fluid

Recovery from the Cisterna.—In experiments (Bhattacharya and Feldberg, 1958b) in which the cerebral ventricles were perfused with plain Locke solution, the effluent collected from the cannulated cisterna magna at the beginning of the perfusion always exceeded the inflow and was greater than 2 ml. in 20 min. As perfusion continued, the volume of the outflow approximated to that of the inflow within 1 or 2 hr. When the perfusion fluid contained acetylcholine 1:200,000 together with an anticholinesterase, or histamine 1:200,000, the outflow almost always exceeded the inflow (Table I). In one experiment (No. 5) it approximated to the inflow within the first hr., in another experiment (No. 3) it remained high although perfusion was continued for over 3 hr. In all other experiments the effluent was collected from the cisterna for up to 2 hr., and although the outflow had decreased by this time it remained greater than the inflow. The finding that the volume of fluid recovered was equal to the volume infused, or even in excess of it, does not mean that substances like acetylcholine or histamine added to the perfusion fluid were fully recovered in the outflowing fluid. The recovery of these substances varied from experiment to experiment independent of the volume of the effluent collected.

In Table I the 7 experiments in which the perfusion fluid contained acetylcholine 1:200,000 together with an anticholinesterase are arranged in such a way as to illustrate the recovery in ascending order. In experiment 1 recovery was between 15 and 20% and in experiment 7 between 95 and 100%. In each experiment the degree of recovery varied only slightly from sample to sample; when recovery was low it remained low, when it was high it remained high. In two short experiments in which histamine 1:200,000 was added to the perfusion fluid the recovery was 71% in one and in the other reached 100% after the first sample.

TABLE I

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Recovery in the effluent from the cisterna or aqueduct of acetylcholine (Expts. 1 to 7) or histamine (Expts. 8 and 9) added to the perfusion fluid in a concentration 1 : 200,000. In experiments 1, 3, 4, 5, and 6 the perfusion fluid contained eserine 1 : 100,000, in experiments 2 and 7 neostigmine 1 : 50,000 as well. The values in **bold type** indicate that the outflow was no longer collected from the cisterna but from the aqueduct. The underlined values were obtained after death in experiments 3 and 4 from the cisterna and in experiments 5 and 6 from the aqueduct.

Volume in ml. and Recovery of Acetylcholine or Histamine as % in Consecutive 20 min. Samples

Expt. 1		Expt. 2		Expt. 3		Expt. 4		Expt. 5		Expt. 6		Expt. 7		Expt. 8		Expt. 9	
ml.	%	ml.	%	ml.	%	ml.	%	ml.	%	ml.	%	ml.	%	ml.	%	ml.	%
2·3	15	2·4	50	2·65	74	2·45	80	2·2	85	2·6	90	2·45	95	2·4	76	2·15	71
2·3	15	2·5	60	2·65	78	2·45	82	2·15	80	2·5	90	2·5	97	2·4	100	2·15	71
2·3	20	2·4	58	2·35	70	2·3	82	2·05	75	2·4	85	2·4	100	2·4	100		
2·2	15	2·25	56	2·45	78	2·2	80	2·05	80	2·4	93	2·45	100				
2·3	20	**2.15**	**90**	2·4	80	2·15	80	2·0	85	2·4	90						
		2.0	**93**	2·45	78	2.0	78	2·15	80	**2.1**	**90**						
		2.05	**93**	2·55	78	2.0	98	2·15	80	**2.2**	**93**						
				2·4	78	1.95	100	**2.05**	**95**	**2.05**	**95**						
				2·3	78			**2.15**	**90**	**2.1**	**97**						
				2·45	75			**2.15**	**95**	1.95	100						
				1.95	100			2.05	100								
				1.95	98												

Recovery from the Aqueduct.—In experiments in which perfusion was carried out with Locke solution the outflow from the aqueduct was either equal to the inflow or exceeded it by 0.05 to 0.15 ml. in 20 min. (Bhattacharya and Feldberg, 1958b). The same result was obtained when the ventricles were perfused with Locke solution containing acetylcholine and an anticholinesterase. This is shown in experiments 2, 5, and 6 in Table I. In these experiments the effluent was first collected from the cisterna but later from the aqueduct. This switch-over increased the recovery of acetylcholine to between 90 and 95%. Experiment No. 2 is particularly striking. The recovery during the collection from the cisterna was 50 to 60%. When collection was from the aqueduct, the rate of outflow decreased, but in spite of this reduction the recovery rose to 93%.

Recovery of Acetylcholine after Death.—In some experiments the cat was killed with intravenous pentobarbitone sodium and the effluent was collected from the cannulated cisterna. The precaution was taken to make certain that the free opening of the cannula from which the fluid dripped lay below the level of the spinal cord. When the opening was above the level of the spinal cord the outflow not only stopped on killing the cat but the fluid in the cannula was drawn in and it took several minutes before the cannula filled again and the outflow started. The rate was then equal to the inflow. The initial stoppage and backflow is easily explained by the increased capacity of the subarachnoidal space when the filled vessels collapse after death. When the outflow was from a level below the spinal canal this stoppage and backflow did not occur and the outflow became almost equal to the inflow as illustrated in experiments 3 and 4 of Table I. When collection was from the aqueduct, the level at which the opening of the outflow cannula was kept was immaterial ; the outflow became almost equal to the inflow, and changed little or not at all as illustrated in experiments 5 and 6 of Table I.

When the perfusion with acetylcholine was continued after death the recovery rose either at once, or after one sample, to 100% or to nearly 100% whether the collection was from the cisterna or from the aqueduct. This is illustrated in the experiments 3, 4, 5 and 6 of Table I.

Appearance of Acetylcholine in the Effluent

When the ventricular spaces were perfused with Locke solution and no anticholinesterase was injected into the blood stream, the effluent contained no detectable amounts of acetylcholine when tested on the eserinized leech muscle preparation which responded to acetylcholine 1×10^{-9}.

Effect of Anticholinesterases Injected into the Blood Stream.—When eserine or neostigmine were injected intravenously, or dyflos into both carotid arteries, but no anticholinesterase was added to the perfusion fluid, minute amounts of acetylcholine appeared in the effluent collected from the cisterna. This is shown in Table II. In experiment 7 (Table II), perfusion was continued for over 4 hr., and although the dyflos was injected only once, at the beginning of the perfusion, the output of acetylcholine rose steadily.

TABLE II

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA. APPEARANCE OF ACETYLCHOLINE IN CISTERNAL EFFLUENT AFTER INTRAVENOUS INJECTIONS OF ESERINE OR NEOSTIGMINE OR AFTER INTRACAROTID INJECTIONS OF DYFLOS

	Acetylcholine (ng./min.) in Successive 20 min. Samples of Effluent						
Expt. 1	2	3	4	5	6	7	
	Eserine		Neostigmine		Dyflos		
Nil	0·3	0·4	0·1	0·2	<0·3	0·3	
,,	0·3	0·2	0·2	0·2	<0·3	0·4	
<0·4	0·3	0·5	0·1	0·2	<0·3	0·4	
<0·4	0·3	0·5	0·1	0·2	<0·3	0·6	
			0·1	0·2	<0·3	0·6	
				0·3		1·0	
				0·5		0·8	
				0·6		1·0	
				0·3		1·3	
						1·6	
						1·6	
						1·6	
						2·0	
						1·7	

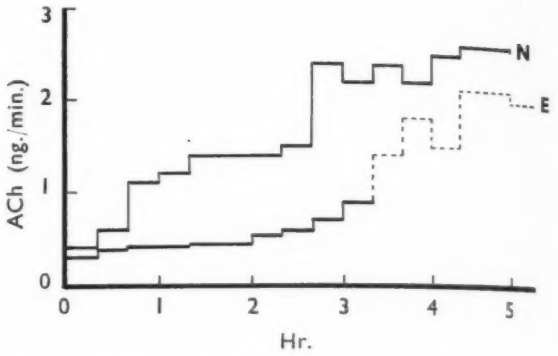

FIG. 1.—Perfusion from the lateral cerebral ventricle in two cats under chloralose anaesthesia. Appearance of acetylcholine in the cisternal effluent on perfusion with eserine (E) or neostigmine (N) in a concentration of 1 : 200,000. The dotted line in experiment E indicates that after 200 min. perfusion eserine was injected intravenously every 20 min., 1 mg. being the first dose and 0.5 mg. at all subsequent injections whilst perfusion with eserine continued. Abscissa: time of perfusion in hr. Ordinate: output of acetylcholine in ng./min.

Effect of Anticholinesterases Added to the Perfusion Fluid.—When an anticholinesterase was added to the perfusion fluid acetylcholine appeared in the effluent from the cisterna, and the amounts varied with the anticholinesterase and with the concentration used for perfusion.

The amounts of acetylcholine which appeared in successive 20 min. samples during perfusion with eserine 1 : 200,000 expressed in ng./min. are shown in Fig. 1. The output did not rise to more than 0.4 ng./min. during the first 2 hr. of perfusion. It was therefore of the same order as that obtained after intravenous injections of eserine. When perfusion was continued for over 3 hr. the acetylcholine output rose to 0.9 ng./min. In experiments in which the concentration of eserine in the perfusion fluid was 1 : 50,000, the amounts of acetylcholine in the cisternal effluent were only slightly greater (see experiment E in Fig. 2 and Table III).

The output of acetylcholine was greater when neostigmine instead of eserine was added to the perfusion fluid. Fig. 1 shows the difference between the effects of the two anticholinesterases when perfused at a concentration of 1 : 200,000. The output after 4 hr. perfusion with neostigmine reached about 2.5 ng./min. This high output of acetylcholine was never obtained when perfusing with eserine 1 : 200,000 or 1 : 50,000 ; it was also greater than the output which occurred when neostigmine was injected intravenously.

TABLE III

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance of acetylcholine (ng./min.) in successive 20 min. samples of cisternal effluent on perfusion with either eserine or neostigmine 1 : 50,000.

	Eserine							Neostigmine																	
Expt. 1	2	3	4	5	6	Average	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Average
<0·4	0·6	0·6	0·4	<0·3	0·3	0·4	1·0	0·6	0·4	0·6	0·4	0·4	0·9	0·4	0·4	1·1	0·5	1·0	0·4	0·5	0·6	1·0	2·2	0·8	0·6	0·7
<0·4	0·8	0·6	0·7	0·4	0·4	0·5	1·0	1·2	1·2	1·1	2·0	1·2	2·0	0·7	1·2	2·0	1·4	2·0	2·3	1·3	1·4	2·3	—	1·2	2·0	1·5
<0·4	1·4	0·5	0·6	0·4	0·7	0·7	1·1	1·8	1·7	1·6	5·1	1·4	2·0	1·0	2·0	2·2	2·2	2·2	2·2	1·6	3·7	4·3	4·0	1·9	2·1	2·4
0·4	1·4	0·5	0·7	0·7	0·8	0·7		1·3	1·8	1·6	4·4	2·0	2·2	1·2	1·8	2·0	3·5	2·9	3·2	2·5	3·9	7·1	5·0	2·8	2·2	3·0
0·5	1·5	0·5	0·7	0·5	0·7	0·7				1·8	4·2	2·3	3·1	1·6	3·3	2·4	2·8	4·2	3·7	2·5	4·2	5·8	6·2	3·7	2·2	3·4
0·8	1·5	0·5	0·8	0·7	1·1	0·9				2·3	4·0	2·7	2·5	1·7	3·7	3·6	3·3	6·0	3·2	2·7	4·0	5·2	7·1	4·0	2·3	3·6
0·8	1·2	0·5	1·0	—	1·0	0·9										4·0	3·0	5·0	3·0	3·0	4·7	4·3	7·6	5·0	2·9	4·3
0·6	1·7	0·5	1·3	0·9	1·0	1·0											2·8	5·1	3·8	3·3	4·0	3·8	6·1	7·1	3·2	4·4
		0·7	1·5	0·7	1·1	1·0												6·0	6·1	4·7	4·4	4·3	6·6	7·3	4·9	5·5
				0·7	1·0	0·8															4·5	3·8	7·1	7·6	4·8	5·6
				1·0	0·8	0·9																5·8	8·7	7·0	5·2	6·7
				0·8	0·9	0·8																	8·4	6·2	6·1	6·9
					1·2	1·2																	8·5	6·8	6·5	7·3
																								6·2	5·5	5·9
																								6·2	5·0	5·6
																								6·6	5·8	6·2
																								6·2		6·2

When neostigmine 1:50,000 instead of 1:200,000 was perfused the output of acetylcholine in the cisternal effluent increased further (Fig. 2; Table III). From the average output of acetylcholine in successive 20 min. samples obtained in the 6 experiments with eserine and in the 19 with neostigmine recorded in Table III, it can be seen that, after 1 to 2 hr. perfusion with neostigmine 1:50,000, the output was more than 4 times greater and after longer perfusion even 7 to 8 times greater than after perfusion with eserine 1:50,000. Thus the difference between neostigmine and eserine became more pronounced as the perfusion continued. The greater efficacy of neostigmine is shown in the same experiment. For instance in an experiment in which perfusion during the first 4 hr. was with eserine 1:50,000 (Fig. 3), the output reached about 1 ng./min. during the second hour and then remained relatively steady, a value of 1.2 ng./min. being obtained during the last 20 min. of the 4 hr. period. The perfusion fluid was then changed to one containing neostigmine 1:50,000 and the output rose steeply and reached a value of 5.5 ng./min. within 2 hr.

The amounts of acetylcholine which appeared in successive 20 min. samples of cisternal effluent on perfusion with dyflos 1:50,000 were of the same order as after perfusion with eserine 1:50,000 as illustrated in Fig. 2, although on a weight for weight basis eserine is about 35 times more potent in inhibiting true cholinesterase than dyflos, whereas dyflos is about four times more potent in inhibiting pseudocholinesterase than eserine (Bhattacharya and Feldberg, 1958a).

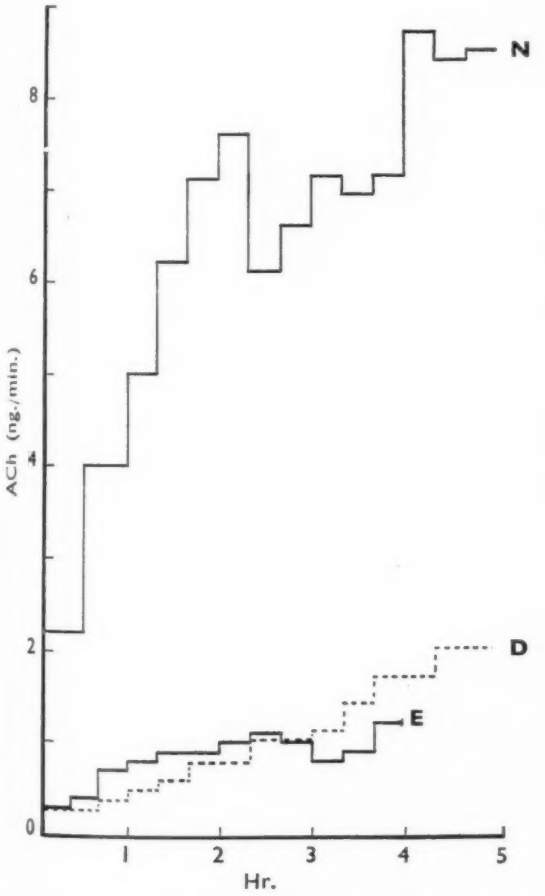

FIG. 2.—Perfusion from the lateral cerebral ventricle in three cats under chloralose anaesthesia. Appearance of acetylcholine in the cisternal effluent on perfusion with eserine (E), with neostigmine (N) and with dyflos (D) in a concentration 1:50,000. Abscissa: time of perfusion in hr. Ordinate: output of acetylcholine in ng./min.

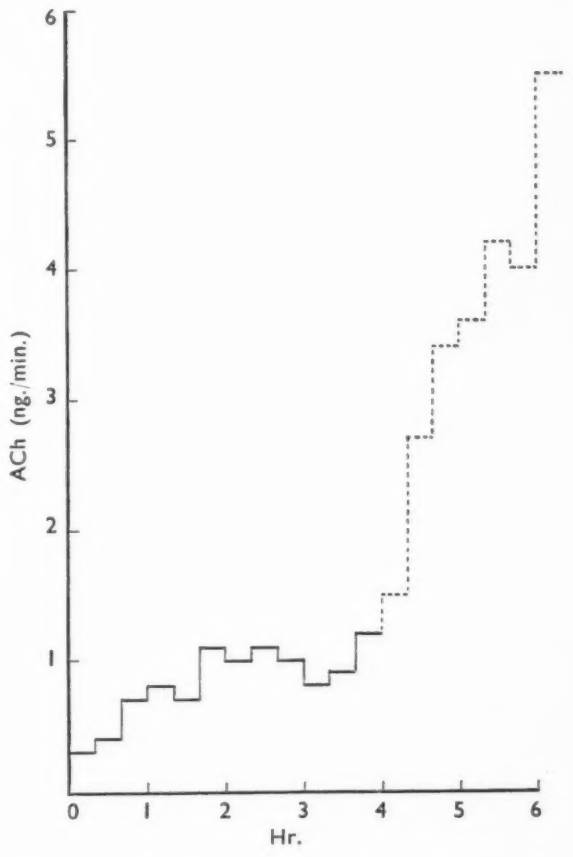

FIG. 3.—Perfusion from the lateral cerebral ventricle in a cat under chloralose anaesthesia. Perfusion during the first 4 hr. was with eserine 1:50,000 (continuous line) and afterwards with neostigmine 1:50,000 (broken line). Appearance of acetylcholine in cisternal effluent. Abscissa: time of perfusion in hr. Ordinate: output of acetylcholine in ng./min.

Effect of Anticholinesterases Added to the Perfusion Fluid and Injected into the Blood Stream.—If acetylcholine were to diffuse into the ventricles and into the subarachnoidal space from the tissue bordering these spaces only, no increase in the output of acetylcholine by additional intravenous injection would be expected, particularly in experiments in which a strong concentration of neostigmine is used for perfusion, because the cholinesterase of the structures in immediate contact with the ventricular spaces must be assumed to be fully inhibited. Thus the additional intravenous injection of an anticholinesterase would not be able to improve the conditions for the escape of acetylcholine into the ventricular or subarachnoidal spaces. On the other hand, if acetylcholine were able to diffuse into the perfusing fluid from more distant regions, the additional intravenous injection of an anticholinesterase might allow this acetylcholine to reach the fluid undestroyed, thereby increasing the output beyond that obtained when the anticholinesterase is added to the perfusion fluid only. The results obtained varied according to whether eserine or neostigmine was used for intravenous injection. Eserine increased the output of acetylcholine; the effect of neostigmine was doubtful.

The experiment illustrated in Fig. 1 shows the increased output of acetylcholine which occurred on intravenous injection of eserine during perfusion with eserine 1:200,000. Table IV gives the results of experiments in which the effects of intravenous injections of eserine or neostigmine were studied during perfusion with either anticholinesterase in a concentration of 1:50,000. In the first four experiments of Table IV, perfusion was continued for several hours with eserine 1:50,000 before intravenous injections of either eserine or neostigmine were given. The output of acetylcholine, which had reached a relatively steady level before these injections, rose in all four experiments. Nevertheless the output remained lower than that obtained in many experiments in which neostigmine 1:50,000 was used for perfusion and no additional intravenous injections were given. In experiments 5 to 12 of Table IV perfusion was with neostigmine 1:50,000. As the output of acetylcholine usually increased on prolonged perfusion with this concentration of neostigmine it was necessary to find out whether the increases which occurred after the additional intravenous injections of neostigmine or eserine were the outcome of these injections. The result is shown in Fig. 4, in which the continuous line represents the mean output of acetylcholine for each successive 20 min. sample from the 19 experiments of Table III in which perfusion was with neostigmine 1:50,000. The individual values obtained from experiments 5 to 12 of Table IV after intravenous neostigmine are shown as solid circles, those obtained after intravenous eserine as squares. It will be seen that the solid circles are grouped around the continuous line, about half of them above and half of them below. This shows that the intravenous injection of neostigmine had not significantly changed the output of acetylcholine. On the other hand, most of the squares lie well above the continuous line, which shows that the intravenous injection of eserine had increased the output of acetylcholine in the effluent.

TABLE IV

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance of acetylcholine in cisternal effluent during perfusion with eserine or neostigmine 1: 50,000. The values in **bold type** were obtained when either eserine or neostigmine was given intravenously as well. In Expt. 13 collection was from the aqueduct.

Expt. No.	Anticholinesterase Used for		Acetylcholine (ng./min.) in Successive 20 min. Samples of Effluent																
	Perfusion	Intravenous Injection																	
1	Eserine	Eserine	<0·3	0·4	0·4	0·7	0·5	0·7	—	0·9	0·7	0·7	1·0	0·8	1.9	2.0	1.6	1.5	1.7 1.7
2	,,	,,	0·6	0·6	0·5	0·5	0·5	0·5	0·5	0·5	0·7	2.3							
3	,,	,,	0·6	0·8	1·4	1·4	1·5	1·5	1·2	1·7	2.9	2.4	4.2	4.7	3.8	4.3	4.4		
4	,,	Neostigmine	0·4	0·7	0·6	0·7	0·7	0·8	1·0	1·3	1.5	2.2	2.2	2.7	4.0	3.6	3.6		
5	Neostigmine	,,	0·6	1·1	1·6	1·6	1·8	2·3	2.5	4.4	5.5	5.7	6.1	5.0	5.2				
6	,,	,,	0·4	2·0	5·1	4·4	4·2	4·0	4.5	4.6	4.6	5.2	6.5	4.8	7.4	6.5	6.6		
7	,,	,,	0·4	1·2	1·4	2·0	2·3	2·7	3.4	5.5	6.6	6.6	6.6	8.9	8.5	9.6	10.0		
8	,,	Eserine	1·0	1·0	1·1	3.1	4.9	5.9	5.3	7.6	6.9								
9	,,	,,	0·6	1·2	1·8	1·3	—	3·9	7.0	7.1	8.3	10.5	11.3	11.0					
10	,,	,,	0·4	1·2	1·7	1·8	2.3	6.5	7.8	7.0	7.5	8.4	7.9	7.3					
11	,,	,,	0·5	1·4	2·2	3·5	2·8	3·3	3·0	2·8	9.8	12.0	12.0	11.6	11.6	11.6	11.6	14.5	
12	,,	,,	2.0	3.0	4.4	4.7	6.9	7.6	7.7	8.5	8.1	7.1	7.5	7.1	7.6	10.0	10.3		
13	,,	,,	0·4	0·8	1·0	1·4	1·1	1·6	1·5	1·9	6.8	7.1	8.4	9.1	7.0				

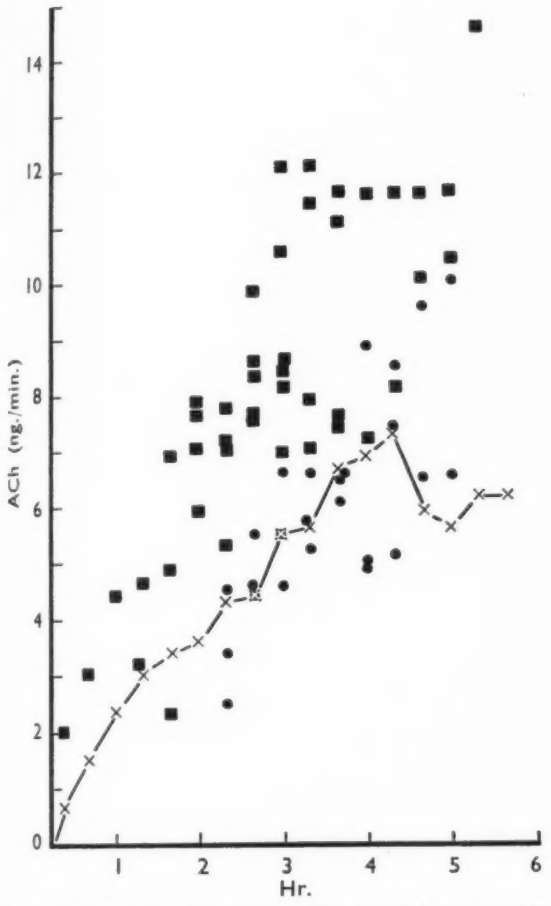

FIG. 4.—Perfusion from the lateral cerebral ventricle in cats under chloralose anaesthesia. Appearance of acetylcholine in the cisternal effluent in successive 20 min. samples during perfusion with neostigmine 1: 50,000. Continuous line: mean output of the 19 experiments recorded in Table III. Individual values for each 20 min. sample after intravenous neostigmine (●) or intravenous eserine (■) are shown. Abscissa: time of perfusion in hr. Ordinate: output of acetylcholine in ng./min.

Collection from the Aqueduct.—When the collection was from the aqueduct, acetylcholine continued to appear in the effluent. This is shown in the experiments of Table V in which neostigmine 1:50,000 was added to the perfusion fluid. The output of acetylcholine increased in successive samples, but the increase, particularly during prolonged perfusion, was smaller than that in most of the corresponding experiments of Table III with the collection from the cisterna. It would therefore seem that a relatively large portion of the acetylcholine which appears in the effluent collected from the cisterna does not enter the perfusion fluid in the lateral and third ventricles. However, the results of the following three experiments led to a somewhat different conclusion. In two experiments in which perfusion was with neostigmine 1:50,000, collection was first from the cisterna and later from the aqueduct. The results are given in Table VI. The change of collection did not decrease the output of acetylcholine which had gradually risen in the course of 2 hr. of perfusion; the output continued to rise, although the rise may have been more gradual than it would have been if collection had continued from the cisterna. In another experiment (Expt. 13 of Table IV) in which perfusion was also with neostigmine 1:50,000, collection was from the beginning from the aqueduct. The output of acetylcholine had risen to less than 2 ng./min. within 3 hr., but when eserine was then injected intravenously the output rose quickly to values between 7 and 9 ng./min.

TABLE V

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance of acetylcholine in effluent from the aqueduct during perfusion with neostigmine 1: 50,000.

Expt. No.	Acetylcholine (ng./min.) in Successive 20 min. Samples of Effluent													
1	0·7	1·8	2·6	2·1	4·2	2·5	3·5	3·7	2·4	2·6	2·9	3·1		
2	1·1	1·7	1·8	2·4	2·3	2·4	1·6	4·4	5·0	4·4	5·8			
3	0·7	0·9	0·9	0·8	1·0	1·4	1·3	1·3	1·1	1·3	1·5	2·4	2·5	3·3

TABLE VI

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance of acetylcholine in effluent from the cisterna and later from the aqueduct (numerals in **bold type**) during perfusion with neostigmine 1: 50,000.

Expt. No.	Acetylcholine (ng./min.) in Successive 20 min. Samples of Effluent												
1	1·1	2·0	2·2	2·0	2·4	3·6	4·0	**4.3**	**4.3**	**5.0**	**4.8**	**4.3**	**5.1**
2	0·4	1·2	2·0	1·8	3·3	3·7	**4.1**	**5.9**	**5.1**	**5.9**	**5.0**	**6.3**	**6.2**

Output after Death.—A few cats were killed by intravenous injection of pentobarbitone sodium several hours after the perfusion with neostigmine 1:50,000 when the acetylcholine in the effluent from the cisterna had reached a high level. Perfusion was continued. Killing the cat resulted in a progressive steep decrease of the acetylcholine output from sample to sample. This is illustrated by two typical experiments in Fig. 5. In experiment A the cat was killed after 3 hr. perfusion when the acetylcholine output had reached a level of 4.7 ng./min. In this experiment there was a further slight rise to 5.2 ng./min. in the first 20 min. sample collected after the pentobarbitone injection, then a fall to 2.6 and 0.6 ng./min. in the following two samples and finally a fall to 0.3 ng./min. Usually the fall occurred in the first

sample collected after killing the cat as in experiment B of Fig. 5. In this experiment neostigmine was injected intravenously after 2 hr. perfusion and the acetylcholine output in the following 200 min. reached a value of 10.6 ng./min. before the cat was killed; the output then fell to 2.3 ng./min. within 1 hr.

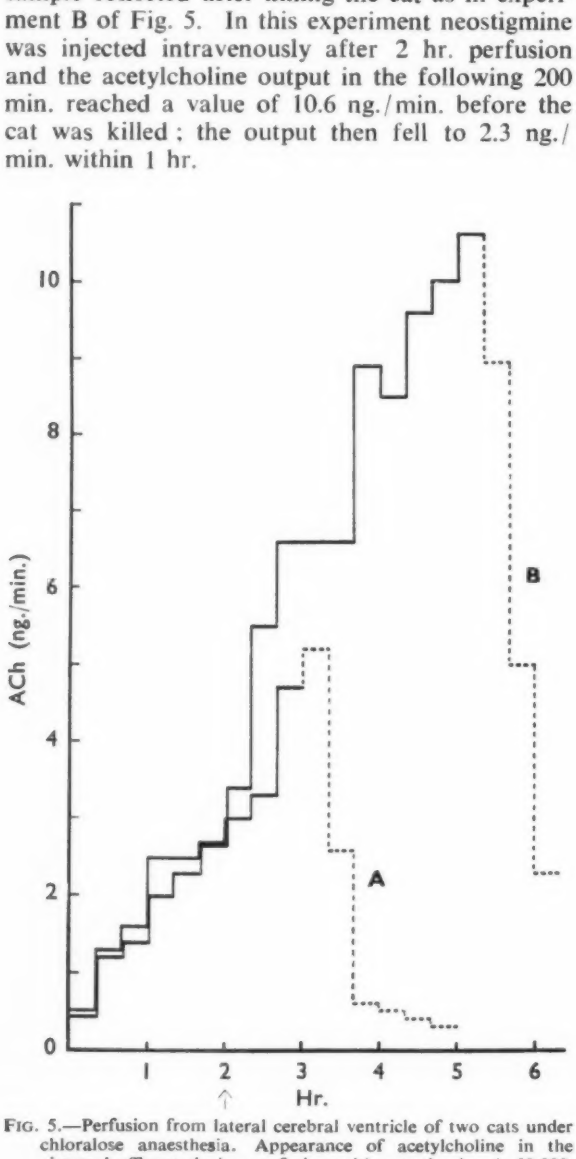

FIG. 5.—Perfusion from lateral cerebral ventricle of two cats under chloralose anaesthesia. Appearance of acetylcholine in the cisternal effluent during perfusion with neostigmine 1: 50,000. Continuous line perfusion in living cat: broken line perfusion after death due to intravenous pentobarbitone sodium. The arrow refers to the upper tracing and indicates the beginning of intravenous injections of neostigmine at 20 min. intervals. Abscissa: time of perfusion in hr. Ordinate: output of acetylcholine in ng./min.

Passage of Anticholinesterases from the Blood Stream into the Perfusion Fluid

When the anticholinesterases were injected into the blood stream tubocurarine was injected as well. Since the assay of the effluent for anticholinesterase involved measuring acetylcholine contractions on the frog rectus muscle, and since tubocurarine is known to depress these contractions, it was necessary to find out if some of the injected tubocurarine appeared in the perfusion fluid and if it would interfere with the assay.

The contractions produced by 1 μg. acetylcholine in the frog rectus muscle suspended in a 5 ml. bath were slightly depressed by 0.1 μg. tubocurarine. However, no depression occurred if the acetylcholine was given with 4 ml. perfusate collected during 40 min. after an intravenous injection of 1 to 10 mg./kg. tubocurarine. This means that tubocurarine had not entered the perfusion fluid, or at least at a rate of less than 2.5 ng./min. which would not interfere with the assay.

Eserine and Neostigmine.—In Table VII the appearance of eserine and neostigmine in the cisternal effluent collected in successive 40 min.

TABLE VII

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance in the effluent from the cisterna or from the aqueduct (values in **bold type**) of anticholinesterases after their intravenous injection.

Anticholinesterase (ng. min.) in Successive 40 min. Samples of Effluent												
Expt. No.												
1	2	3	4	5	6	7	8	9	10	11	12	13
Eserine								Neostigmine			Dyflos	
24	40	10	36	53	28	25	25	0·5	1·1	2·6	2·1	1·8
48	26	43	52	28	29	—	21	0·4	1·0	2·5	3·1	2·5
61	20	35	83	34	37	30	24	0 4	1·0	3·4	3·1	4·0
75	18	27	92	**21**	**16**	**15**	**10**	0 8	2·2	6·4	3·1	3·7
	20	28	92	**20**	**18**	—	**10**		1·8	6·7		
		31	63	**20**	**16**	**17**	**10**					
					17	**16**						

samples is shown. In all experiments the anticholinesterases were injected intravenously at the beginning of the collection and again at 20 min. intervals. First 1 mg. and thereafter 0.25 mg. were injected. It will be seen that eserine appeared in much larger amounts in the effluent than neostigmine. The mean value of all samples collected from the cisterna in the experiments of Table VII was 39 ng./min. for eserine and 2.2 ng./min. for neostigmine. In four of the eserine experiments the effluent was later collected from the aqueduct. This decreased the content of eserine in the effluent to about half its previous value; therefore about half of the eserine assayed in the cisternal effluent has entered the perfusion fluid on its passage through the lateral and third ventricles.

Dyflos.—The last two experiments of Table VII show the amounts of dyflos found in the cisternal effluent when the drug was injected intravenously

in amounts (first 1.75 mg. and thereafter a quarter of this dose) somewhat higher than the amounts of eserine and neostigmine used in the corresponding experiments. The passage of dyflos into the perfusion fluid is of about the same order as that of neostigmine and much lower than that of eserine. Since dyflos is known to be an irreversible inhibitor of cholinesterase, it was thought that when injected intravenously part of it might have entered into a more or less irreversible combination with the pseudocholinesterase of the blood before reaching the brain. Therefore in three experiments 1 mg./kg. dyflos was injected into each carotid artery. In the first two experiments the injections were made immediately before collection of the first sample was begun. In the third experiment the injections were made before perfusion was started ; about 10 min. later 1 ml. of cerebrospinal fluid was collected, perfusion was then started and the first sample collected. The results of these three experiments are given in Table VIII. The amounts of dyflos which

TABLE VIII

PERFUSION FROM THE LATERAL CEREBRAL VENTRICLE IN CATS UNDER CHLORALOSE ANAESTHESIA

Appearance in cisternal effluent of dyflos after its injection into the carotid artery. At the start of the collection of sample 5 a second injection of 2 mg./kg. dyflos was made into each carotid.

Sample	Time of Collection in min.	Dyflos (ng./min.) in Effluent		
		Expt. 1	Expt. 2	Expt. 3
1	20	4	2·5	3
2	20	1	2	3
3	40	0·2	0·8	2
4	40	0·05	0·3	1
5	20			9
6	40			5

passed into the cisternal effluent during the first 20 min. after the carotid injections were between 2.5 and 4 ng./min., and were of the same order as those found in the experiments with intravenous dyflos. The 1 ml. of cerebrospinal fluid collected at the beginning of the third experiment contained 50 ng. which corresponds to an output of 5 ng./min. The amounts of dyflos diminished in each successive sample, but when, as in experiment 3, the drug was injected a second time and in a larger amount (2 mg./kg. into each carotid artery), the output increased in the next sample to 9 ng./min. and then decreased again.

DISCUSSION

Recovery of Substances Added to the Perfusion Fluid.—Acetylcholine added to the fluid perfusing the cerebral ventricles and protected by an anticholinesterase was incompletely recovered in the cisternal effluent in spite of the fact that the outflow was greater than the inflow. Therefore an outflow that exceeds the inflow should not be taken as an indication of full recovery of substances dissolved in the perfusion fluid, or even of the fluid itself. The incomplete recovery could be explained in different ways. Substances could either be absorbed from the choroid plexuses, be taken up by the ependyma, could diffuse into the brain substance or could escape into more distant regions of the subarachnoidal space where they would mix with the cerebrospinal fluid and be gradually absorbed into the blood stream.

The finding that the recovery of acetylcholine in the effluent from the aqueduct was always 90% or more renders it unlikely that selective absorption from the choroid plexuses is the main cause for the low and irregular recovery from the cisternal effluent, because the third and lateral ventricles with their choroid plexuses are still included when collection is from the aqueduct. For similar reasons it is unlikely that the unrecovered acetylcholine has been taken up by the ependyma. It is also unlikely that the incomplete and irregular recovery is mainly the result of diffusion into the brain substance because this would imply that diffusion varies greatly from brain to brain. It seems therefore more likely that acetylcholine escapes into distant parts of the subarachnoidal space. The absence of a foramen of Magendie in cats means that the fluid leaving the ventricle through the foramina of Luschka has to traverse the subarachnoidal space surrounding the brain stem before reaching the cisterna. Some mixing with the cerebrospinal fluid is thus inevitable and some of this mixed fluid is probably carried away into the subarachnoidal space which covers the cerebrum and is absorbed into the blood stream. The devious route the perfusion fluid has to take and the fact that on its way to the cisterna it comes into contact with the main current of cerebrospinal fluid make it understandable why the loss of acetylcholine in the cisternal effluent varies so much from experiment to experiment. After death, when the circulation of cerebrospinal fluid has ceased, the only outlet for the perfusion fluid is through the cannulated cisterna and this would explain why there is then 100% recovery of acetylcholine. The finding that, in the living cat, the recovery in the effluent from the aqueduct is not complete, although it is over 90%, means that some acetylcholine is lost when the fluid passes the ventricular spaces and therefore the possibility of some absorption by the choroid plexus or removal by the ependymal cells cannot be

excluded. There is some evidence that substances present in the cerebrospinal fluid can be taken up by the ependyma and the nervous tissue surrounding the ventricles and the subarachnoidal space (Wallace and Brodie, 1940 ; Bakay and Lindberg, 1949 ; Lindberg and Ernster, 1950 ; Sacks and Culbreth, 1951 ; Rodriguez, 1955).

Appearance of Acetylcholine in the Perfusion Fluid.—The acetylcholine detected in the effluent cannot have originated from acetylcholine circulating in the blood stream, because it appeared not only when anticholinesterases were injected intravenously but also, and in even greater amounts, when they were added to the perfusion fluid. Therefore the acetylcholine must derive from the brain. Further, the finding that after death there was a steep fall in the output of acetylcholine excludes a simple process of leakage from brain tissue and suggests central cholinergic neuronal activity as the cause for the appearance of acetylcholine in the effluent.

There are a few previous reports on the appearance of acetylcholine in the cerebrospinal fluid collected from the cisterna (Feldberg and Schriever, 1936 ; Chang, Hsieh, Li and Lim, 1938 ; Adam, McKail, Obrador, and Wilson, 1938). In these experiments also anticholinesterases were given either by injection into the blood stream or by addition to the fluid with which the cerebral ventricles were perfused or by a combination of both. Further, MacIntosh and Oborin (1953) showed that acetylcholine diffuses out of an eserinized surface in the brain of the cat.

Since the anticholinesterases present in the perfusion fluid are unlikely to penetrate deeply into the brain substance the acetylcholine of the effluent must originate from structures close to the surface over which this fluid passes. On prolonged perfusion the layer of brain tissue into which the anticholinesterases penetrate may, however, increase and the acetylcholine may diffuse into the fluid from deeper layers. This would explain why the acetylcholine content of the effluent increases on prolonged perfusion. The further increase obtained when eserine was injected intravenously during perfusion with neostigmine could be explained by diffusion from even more distant layers, because eserine given intravenously would naturally cause inhibition, or at least partial inhibition, of the cholinesterase in the whole brain.

The acetylcholine appearing in the effluent from the aqueduct must have entered the perfusion fluid on its passage through the lateral and third ventricles. The structures of origin are most likely to be the superficial layers of the caudate nucleus, the thalamus and some of the hypothalamic structures which are known to be rich in acetylcholine and choline acetylase, and which form part of the walls of the ventricles. Acetylcholine, however, is added to the cisternal effluent when the fluid passes through the fourth ventricle, or through the subarachnoidal space around the brain stem. In some experiments this contribution was only small.

The finding that greater amounts of acetylcholine appear in the perfusion fluid when it contains neostigmine instead of eserine cannot be explained by a greater potency of neostigmine as an anticholinesterase. If the greater output of acetylcholine nevertheless signifies greater inhibition of brain cholinesterase, it must mean that, when the blood-brain barrier is circumvented, neostigmine has a greater ability to penetrate to the actual sites of cholinesterase, and once having penetrated to these sites becomes more firmly attached than eserine. The greater ability of neostigmine to penetrate to these sites could be explained by a greater effect of neostigmine on the permeability of the nervous tissue. In this connexion it is of interest that neostigmine greatly reduces the excitability of the sciatic nerve whereas eserine does so to a lesser extent. This effect is attributed not to inhibition of cholinesterase but to interference with the permeability changes during nerve activity (Zaimis, 1953, and personal communication). Easier penetration or firmer attachment of neostigmine would also explain why the acetylcholine output rises more steeply on prolonged perfusion with neostigmine than with eserine.

Desmedt and La Grutta (1957) suggested that the arousal reaction of the sleeping brain produced in the *encéphale isolé* by carotid injections of anticholinesterases was related to inactivation of pseudocholinesterase rather than to inactivation of true cholinesterase in the brain, because selective inhibitors of pseudocholinesterase were more effective than those of true cholinesterase. The finding that on perfusion with neostigmine instead of eserine the output of acetylcholine was greater cannot be explained on these lines because neostigmine is a less potent inhibitor of pseudocholinesterase than eserine. Further, if the output of acetylcholine were related to inactivation of brain pseudocholinesterase it should be greater with dyflos than with neostigmine, because dyflos is a selective inhibitor of pseudocholinesterase and is active on this enzyme in smaller amounts than neostigmine (Bhattacharya and Feldberg, 1958a).

However, the output of acetylcholine on perfusion with dyflos was less than on perfusion with neostigmine.

The greater output of acetylcholine on perfusion with neostigmine instead of eserine emphasizes the fact that the potency of an anticholinesterase on cholinesterase preparation *in vitro* is not a reliable index for its pharmacological actions or its ability to inhibit the destruction of acetylcholine *in vivo*. Similar conclusions were reached when discussing the action of these two anticholinesterases on the leech muscle (Bhattacharya and Feldberg, 1958a).

Passage into the Perfusion Fluid of Substances Injected into the Blood Stream.—The finding that anticholinesterases injected into the blood stream pass into the perfusion fluid, although in varying amounts, and can be assayed in the cisternal effluent could be the result either of passage through the choroid plexuses or of diffusion from the brain tissue. If the anticholinesterases passed into the perfusion fluid from the choroid plexuses the amounts assayed in the effluent from the aqueduct should be only a little less than those assayed in the cisternal effluent. In both conditions the choroid plexuses of the third and lateral ventricles are included in the perfusion, the only difference being that the contribution from the choroid plexus of the fourth ventricle is lacking when collection is from the aqueduct. However, when in the course of an experiment collection from the cisterna was changed to that from the aqueduct the amounts of eserine assayed in the effluent decreased to about half. This suggests an extrachoroidal origin, and diffusion from the brain substance into the perfusion fluid is probably the main mechanism responsible for the appearance of the anticholinesterases in the effluent. From the experiments of MacIntosh and Oborin (1953) we know that such diffusion occurs ; in cats 0.02 ml. fluid/min./cm.^{2} was found to transude from the exposed cerebral cortex into a saline pool created at its surface. For substances present in the blood stream to transude in this way into the ventricular and subarachnoidal spaces they must first have passed the blood-brain barrier. The finding, therefore, that substances injected into the blood stream appear in different amounts in the perfusion fluid is likely to provide a basis for studying quantitatively the passage of pharmacologically active substances through the blood-brain barrier. From the present experiments it would appear that tubocurarine does not pass the blood-brain barrier, and that eserine passes this barrier about 20 times more readily than neostigmine. One should expect therefore that central effects are more prominent with eserine than with neostigmine when these anticholinesterases of almost equal potency are injected into the blood stream. This is in accordance with the following known facts.

Bülbring and Burn (1941) found that eserine was effective on the knee jerk in much smaller doses than neostigmine when these anticholinesterases were injected into the blood perfusing the spinal cord of a dog. Later Eccles, Fatt and Koketsu (1954) and Eccles, Eccles and Fatt (1956) showed the same difference between intravenous eserine and neostigmine on the discharge of the Renshaw cells in the spinal cord. Intravenous eserine greatly prolonged the repetitive discharge evoked by an antidromic volley in the motor neurones and induced spontaneous discharge, whereas intravenous neostigmine was either inactive or had only a slight effect on the evoked discharge. On the other hand, when injected through the recording cannula in the environment of the Renshaw cells, neostigmine was at least as active as eserine. The difference in effectiveness on intravenous injection was attributed to the ability of eserine to pass the blood-brain barrier more readily than neostigmine.

The same explanation could be advanced for the observations that intravenous eserine has a strong, and intravenous neostigmine a weak, action on respiration in anaesthetized and decerebrate cats (Erdmann, Kempe and Lühning, 1955), and that intravenous eserine produces in unanaesthetized cats and monkeys, low-voltage irregular, fast activity in the electroencephalogram similar to the activity pattern of the arousal reaction, whereas intravenous neostigmine does not alter the pattern even when given in doses which cause profound peripheral effects (Funderburk and Case, 1951 ; Bradley, 1953 ; Bradley and Elkes, 1953, 1957). There are also observations of Funderburk and Case (1947) on the effect of anticholinesterases on behaviour in cats ; an intravenous injection of eserine, but not of neostigmine, abolished the conditioned response for as long as 2 hr. In rats, intravenous eserine produces a rise in arterial blood pressure of central origin which is rarely seen with intravenous neostigmine (Varagić, 1955 ; Hornykiewicz and Kobinger, 1956 ; Medaković and Varagić, 1957), but on cisternal injection both anticholinesterases have a strong pressor effect as shown by Hornykiewicz and Kobinger.

Funderburk and Case (1951) quote an unpublished observation by Du Bois, Erway and Byerrum that intravenous eserine increases the

acetylcholine content of the brain of the rat, whereas intravenous neostigmine does not. This observation again is in line with our finding that in experiments in which neostigmine was present in the perfusion fluid, intravenous eserine definitely increased the output of acetylcholine in the effluent whereas the effect of intravenous neostigmine was doubtful. The difference in the ability of the two anticholinesterases to pass the blood-brain barrier would account for these results as well as for those of Du Bois, Erway and Byerrum.

The results obtained on the appearance in the perfusion fluid of intravenously injected dyflos resembled those obtained with neostigmine rather than those with eserine. Since the potency of dyflos in inhibiting true or pseudocholinesterase activity is different from the potency of neostigmine or eserine, its effectiveness in eliciting central reactions on intravenous injection cannot directly be compared with that of the other two anticholinesterases.

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THE RÔLE OF QUININE IN HAEMOLYSIS

BY

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Quinine produces haemolysis of rabbit and human red blood cells in concentrations up to 1 in 700. In a concentration of 1 in 10,000 it increases the degree of haemolysis produced by various haemolytic agents like saponin, bile salts and digitonin *in vitro*. Quinine when injected intravenously in 35 mg./kg. dose in rabbits increases the susceptibility of red blood cells to the haemolytic action of saponin. Intravascular haemolysis following the administration of quinine is seen in cases of blackwater fever, and on the basis of experimental work described in the paper it is suggested that quinine precipitates these haemolytic episodes by rendering the red blood cells more susceptible to the action of tissue lytic factors.

The acid salts of quinine have a haemolytic action on erythrocytes in high concentration (Chopra, Mukerji, and Chopra, 1954), but these concentrations are not attained *in vivo* during the therapeutic use of quinine in cases of malaria. Kligler (1923) showed that dilutions of bile and quinine which separately had no effect on red blood cells caused prompt laking when used together. Mer, Birnbaum, and Kligler (1940) using ox bile as a haemolytic agent showed that the erythrocytes from malarial subjects were haemolysed more readily when quinine was given.

Intravascular haemolysis of erythrocytes during quinine administration is met with clinically in cases of blackwater fever. There is a considerable controversy about the aetiology of blackwater fever. It is now generally accepted that it is caused by infection due to *Plasmodium falciparum* and that administration of quinine and pamaquin precipitates an attack (Fairley and Murgatroyd, 1940). Innumerable instances from the literature can be cited where patients did not get haemoglobinuria until they had received a large dose of quinine; should the patient recover from the effects of the fever, a second dose of quinine may produce haemoglobinuria again. Fairley and Murgatroyd (1940) reported a case where four attacks occurred in one patient following the administration of quinine. Thus quinine under certain circumstances can cause haemolysis, but its mechanism of action has not been elucidated. The present work is an attempt to study the rôle of quinine in modifying the response of red blood cells to various haemolytic agents.

METHOD AND MATERIAL

The degree of haemolysis was estimated by a photoelectric colorimeter. The method is based on the principles of estimating total haemoglobin in a completely haemolysed blood sample and in the supernatant after the addition of different concentrations of haemolytic agents to the blood. The degree of haemolysis is then expressed in terms of total haemoglobin which represents 100% haemolysis; for example, if the total haemoglobin of whole blood is 15 g./100 ml., and if the supernatant after haemolysis gives a figure of 5 g., then the degree of haemolysis is 33.3%. The haemoglobin estimation was done by converting it into acid haematin by adding 0.1 N hydrochloric acid and the intensity of the colour developed was read off from the photoelectric colorimeter. The quantity of haemoglobin corresponding to a particular reading on the photoelectric colorimeter was determined by referring to a calibration curve prepared by plotting photoelectric colorimetric readings (% transmission) against the known concentrations of haemoglobin on a semilogarithmic graph paper. The solution containing a known concentration of haemoglobin was prepared from a blood sample, in which the haemoglobin content was determined by the blood iron method of Wong (1928). When readings were plotted as explained above, a linear relation was obtained between the haemoglobin concentration and the photoelectric colorimeter reading. From this curve the quantity of haemoglobin in a sample of blood could be read off directly when the colorimetric reading was known.

To set up an experiment the haemolytic agent was prepared in various dilutions in Simmel's fluid (sodium chloride 8.2 g., potassium chloride 0.2 g., magnesium chloride 0.2 g., calcium chloride 0.2 g., acid sodium phosphate 0.2 g., sodium bicarbonate 0.05 g., in 1 l.

distilled water). The total volume in each tube was 2.4 ml. In another set of tubes the same concentrations of the haemolytic agent, plus quinine dihydrochloride solution in a dilution of 1 in 10,000, were prepared, the total volume being again 2.4 ml. To all these tubes 0.1 ml. of heparinized blood was added. The tubes were kept at room temperature for 30 min. and were centrifuged at slow speed (1,500 to 2,000 rev./min.) for 15 min. The supernatant contained haemoglobin which was estimated in the form of acid haematin on the photoelectric colorimeter as described above.

Saponin (B.D.H.), digitonin (B.D.H.), bile salts (sodium tauroglycocholate, Bengal Immunity), quinine dihydrochloride (Howard) and heparin (Evans) were used.

Results

The range of haemolytic activity of quinine was determined for both rabbit and human erythrocytes. The percentage haemolysis caused by various concentrations of quinine in rabbit and human red blood cells is shown in Table I. Each

Table I

PERCENTAGE HAEMOLYSIS OF RED CELLS BY QUININE DIHYDROCHLORIDE

Conc. of Quinine	Rabbit	Human
1/100	78·0	73·4
1/200	55·5	68·0
1/300	31·2	59·0
1/400	18·0	48·6
1/500	7·5	31·4
1/600	4·0	12·2
1/700	Nil	3·3
1/800	Nil	Nil

estimate is the average of 10 experiments. From the table it is evident that quinine haemolysed rabbit red blood cells in concentrations up to 1 in 600 and human red blood cells in concentrations up to 1 in 700.

Experiments were next done to find out whether quinine in a concentration of 1 in 10,000 increased the degree of haemolysis produced by saponin. Table II gives the average of 15 experiments using

Table II

PERCENTAGE HAEMOLYSIS OF RABBIT BLOOD PRODUCED BY SAPONININ IN THE PRESENCE OF QUININE DIHYDROCHLORIDE

Conc. of Saponin	% Haemolysis	% Haemolysis in Presence of Quinine 1 in 10,000
1/15,000	77	90
1/20,000	72	85
1/30,000	38	63
1/40,000	16	32

the blood from 12 rabbits. From Table II, it is evident that quinine in 1/16 its haemolytic concentration increased the degree of haemolysis produced by saponin. Thus quinine rendered the cells more susceptible to the haemolytic action of saponin.

The work was extended to find out if quinine in a concentration of 1 in 10,000 modified the response of erythrocytes to other haemolytic agents. Table III gives the average % haemolysis

Table III

PERCENTAGE HAEMOLYSIS OF RABBIT RED CELLS BY BILE SALTS IN THE PRESENCE OF QUININE DIHYDROCHLORIDE

Bile Salt Conc.	% Haemolysis	% Haemolysis with Quinine 1 in 10,000
1/400	95·4	100
1/500	82·6	96·8
1/600	43·6	87·4
1/700	15·6	75·3
1/800	5·9	46·2
1/900	0·5	28

produced by bile salts (sodium tauroglycocholate) in ten rabbits. Table IV gives the average % haemolysis produced by digitonin in six rabbits.

Table IV

PERCENTAGE HAEMOLYSIS OF RABBIT RED CELLS BY DIGITONIN IN THE PRESENCE OF QUININE DIHYDROCHLORIDE

Digitonin Conc.	% Haemolysis	% Haemolysis with Quinine 1 in 10,000
1/100,000	92	100
1/120,000	86	94·2
1/140,000	56·8	79·2
1/160,000	42·5	65·1

The effect of quinine on the degree of haemolysis produced by bile salts on human erythrocytes was also studied. Table V gives the average

Table V

PERCENTAGE HAEMOLYSIS OF HUMAN RED CELLS BY BILE SALTS IN THE PRESENCE OF QUININE DIHYDROCHLORIDE

Bile Salt Conc.	% Haemolysis	% Haemolysis with Quinine 1 in 10,000
1/500	94·1	100·0
1/600	73·7	95·2
1/700	39·6	73·9
1/800	14·5	46·3
1/900	5·9	19·9

results obtained in ten experiments on different human blood samples. It is evident from the table that quinine increases the susceptibility of human erythrocytes to haemolysis *in vitro* in the same way as that of rabbit erythrocytes.

Experiments were done to see if quinine had a similar effect on red cell fragility when it was injected intravenously in a rabbit. The technique

was first to determine the degree of haemolysis by different concentrations of saponin added to blood of the animals *in vitro*. Then quinine dihydrochloride, 35 mg./kg. in 20 ml. of 5% glucose-saline solution, was injected intravenously at a slow rate. The percentage haemolysis produced by the same concentrations of saponin was determined on blood withdrawn ½ hr. and 1½ hr. after the quinine injection.

Table VI gives the average results of observations on 15 rabbits. It is evident that the intravenous injection of quinine increased the average percentage haemolysis produced by saponin. There were differences in the degree of haemolysis produced by a given concentration of saponin in different rabbits, but quinine increased the degree of haemolysis produced by a particular concentration of saponin in all cases except in 3 rabbits, in which quinine produced no increase in haemolysis. It was observed that quinine increased the speed, as well as the degree, of haemolysis.

TABLE VI

PERCENTAGE HAEMOLYSIS OF RABBIT BLOOD BY SAPONIN AFTER INTRAVENOUS INJECTION OF QUININE DIHYDROCHLORIDE, 35 MG./KG.

Saponin Conc.	% Haemolysis Before Quinine	% Haemolysis ½ hr. after Quinine Injection	% Haemolysis 1½ hr. after Quinine Injection
1 20,000	81·3	90·4	96·3
1 25,000	62·4	64·7	82·3
1 30,000	45·9	52·1	69·2
1 35,000	32·5	43·5	65·8
1 40,000	27·3	33·9	53·5

DISCUSSION

The close association between administration of quinine in cases of malaria and occurrence of haemoglobinuria has been frequently noted. Foy and Kondi (1937b) found a high positive correlation between quinine administration and blackwater fever in Macedonia. Maegraith (1946) found a high incidence of haemoglobinuria during oral quinine therapy for malignant tertian malaria in the West African Command in 1941 to 1943. Findlay is quoted (Skipper and Haine, 1945) as stating that incidence of blackwater fever diminished considerably after the introduction of mepacrine in place of quinine for treatment and suppression of malaria. A similar lowering of incidence of haemoglobinuria was seen in the great malaria epidemic of 1942 in Greece when quinine was substituted by mepacrine for treatment of malaria (Foy and Kondi, 1950). This is specially remarkable since the relation between malarial incidence and blackwater fever was constant in previous years.

The factor responsible for the occurrence of intravascular haemolysis in blackwater fever has been postulated as a circulating haemolysin by Foy, Kondi, and Moumjidis (1941). Maegraith, Findlay, and Martin (1943) believe that there is a reduction of the normal inhibitory factor in cases of blackwater fever and that haemolysis is due to a shift to the lytic side of the balance between tissue lytic factor and its inhibitors. Though the factors responsible for causing intravascular haemolysis are known, the rôle of quinine in these haemolytic episodes has not been elucidated. On the basis of the experimental evidence presented in this paper it may be suggested that quinine can increase the degree of haemolysis by rendering the red blood cells more susceptible to the action of haemolytic agents. Recently it has been shown (Kärki, Burn, and Burn, 1957) that quinine diminishes the flux of ions in red cells. After storage for 3 to 5 days at low temperature the concentration of potassium within the cell falls and that of sodium rises. When these cells are incubated at 37° in the presence of glucose the concentration of potassium once more rises and that of sodium falls. These changes were found to be diminished by quinidine. Kärki (1958) has found that other antimalarial compounds have a similar action, though pyrimethamine has not. It is quite possible that this effect of quinidine on the permeability of the red cell membrane is connected with its tendency to favour haemolysis.

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THE PRESSOR EFFECT OF PEPTONE IN THE RAT

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Peptone normally causes a rise of blood pressure in rats. Since the pressor response was not abolished or even diminished by adrenalectomy, it could not have been caused by a release of pressor substances from the adrenal glands. The group of drugs (dibenamine, dihydroergotamine, ergotamine, tolazoline, nicotine and hexamethonium) which antagonize or block the peptone-induced pressor action suggests that the pressor effect may be secondary to a release of sympathin by the stimulation of sympathetic ganglia. Since it was not possible to duplicate the pressor response to peptone by either 48/80 or histamine, the hypothesis that peptone acts by liberating histamine can be ruled out.

In the course of experiments on the effect of peptone on the mast cells of the rat, it was found that, following a slight fall, a rise in blood pressure appeared after an intravenous injection of peptone (Beraldo and Mota, 1953).

It has long been known that a rise in blood pressure may follow the initial fall after an intravenous injection of either histamine or pilocarpine into a spinal cat. The appearance of a secondary rise after injection of those drugs has until recently been generally assumed to be due to liberation of sympathin from the adrenals (Dale and Laidlaw, 1912; Burn and Dale, 1926). This does not fully account for the secondary rise since Root (1951) found that the secondary rise caused by pilocarpine was unaffected by adrenalectomy, whereas Slater and Dresel (1952) observed the persistence of a small secondary histamine rise after adrenalectomy. Recently, Trendelenburg (1955) investigating the factors involved in the appearance of the secondary rise in blood pressure after intravenous injections of histamine and pilocarpine concluded that, while the histamine rise is mainly due to liberation of sympathin from the adrenal gland and only to a minor degree to general stimulation of sympathetic ganglia, the pilocarpine rise, on the other hand, is mainly due to the ganglionic actions of this substance and is thus similar to the histamine rise in the adrenalectomized cat.

The rise in blood pressure which follows the injection of peptone could not be accounted for by the histamine usually present in the peptone, since the peptone used was freed of histamine by treatment with permutit. However, as peptone is a potent histamine releaser (Dragstedt, 1937; Feldberg and O'Connor, 1937; Rocha e Silva, Teixeira and Andrade, 1946), it might be possible that the sympathin could be released by histamine liberated from tissues of the rat.

On the other hand the hypothesis that the liberation of other pressor substances from the rat tissues might account for the rise of blood pressure has also been put forward. Both hypotheses have been tested in the experiments reported in this paper.

METHODS

Male Wistar rats weighing 200 to 400 g. were used. The animals were anaesthetized with urethane (175 mg./100 g. of body weight) injected intramuscularly. After 45 to 60 min. the rat was tied to the operating table. A metal cannula was inserted into the trachea. Both vagi and associated sympathetic trunks were cut and 1 mg. of atropine sulphate was injected subcutaneously. The femoral vein close to the inguinal ligament was cannulated with a hypodermic needle mounted in a polythene tube and connected with a burette containing saline. Heparin (50 to 100 units/100 g. of body weight) was injected through the venous cannula. The carotid artery was cannulated with a hypodermic needle mounted in a polythene tube and connected with a mercury manometer of about 2 to 3 mm. internal diameter by a column of normal saline. All solutions were injected through the venous cannula by means of a 1 ml. tuberculin syringe and were washed in with 0.25 ml. of normal saline.

In a few experiments the spinal-rat preparation was used. The animal was anaesthetized, atropinized, its trachea cannulated and both vagi cut, as described above. Clips were put on both carotid arteries temporarily. The spinal cord was cut close to the second

cervical vertebra and artificial respiration was started. The brain was destroyed by a probe thrust through the foramen magnum. A cannula was put into the carotid artery for recording the blood pressure.

Drugs Used.—Urethane (Riedel, S.A.), 17.5 g./100 ml. solution in distilled water. Heparin (Vitrum), 5% solution (5,000 units/ml.). Witte's peptone (Friedr. Witte, Rostock), 10 and 20% fresh solutions in distilled water. These were acidified (*p*H 5), shaken with permutit (1 g. of permutit/g. of peptone), filtered and neutralized prior to use. This treatment freed the solution of contaminating histamine. For most of the experiments Witte's peptone was used although two other peptone preparations have been employed (Pfanstiehl peptone and Bacteriologic Peptone, Park, Davis). The weights of noradrenaline hydrochloride (Delta Chemical Works), (−)-adrenaline bitartrate (Winthrop-Stearns), nicotine (Schering-Kahlbaum) and 5-hydroxytryptamine creatinine sulphate (Sandoz) are those of the base. The quantities of dibenamine hydrochloride (Smith, Kline, and French), dihydroergotamine methane-sulphonate (Sandoz), hexamethonium bromide (Bistrium, Squibb), the butanolamide of lysergic acid (Methergin, Sandoz), atropine sulphate (Poulenc Freres) and dimethylphenylpiperazinium (DMPP) (Parke, Davis) used are expressed as weights of the salts. Pituitrin (Parke, Davis) 5 units/0.5 ml.

Results

The Action of Peptone on the Arterial Blood Pressure.—When peptone in a dose of 10 to 20 mg. (0.05 to 0.1 ml. of a 20% solution)/100 g. of body weight was injected intravenously in atropinized rats usually the blood pressure rose steeply within a few seconds. The first four or five injections caused a definite pressor response, but, on repetition, the same dose or even greater doses of peptone at 5 to 7 min. intervals elicited little or no response at all (tachyphylaxis). Spontaneous recovery of the capacity to produce a new rise was not observed even when the animal was left resting for 3 hr. before testing again. For the purpose of comparing the pressor effect of peptone, a noradrenaline standard solution was used. In view of the gradual desensitization which developed after repeated injections of peptone, it was not possible to bracket the response of the noradrenaline and that of peptone, but a fairly satisfactory comparison between the pressor response of peptone and noradrenaline could be obtained (Fig. 1).

The rise in blood pressure in a group of 30 rats was comparable to that produced by 1 to 10 μg. of noradrenaline. The magnitude of this increase was apparently not dependent upon the initial blood pressure of the animal. In 3 out of 33 rats, however, the injection of peptone failed to produce the pressor effect. All the 3 non-reactive animals showed typical responses to the injection of noradrenaline.

The Rôle of the Adrenal Glands.—Earlier work by Dale and Laidlaw (1912) and by Burn and Dale (1926) in cats indicated that the adrenal glands might be concerned in the pressor responses to pilocarpine and histamine. This led us to investigate the effect of adrenalectomy on the rise in blood pressure following the injection of peptone in the rat. In 8 rats an incision was made in the dorsal region and both adrenal glands excised; 15 to 20 min. later the arterial blood pressure was recorded as described above. A test dose of peptone was injected and the magnitude of

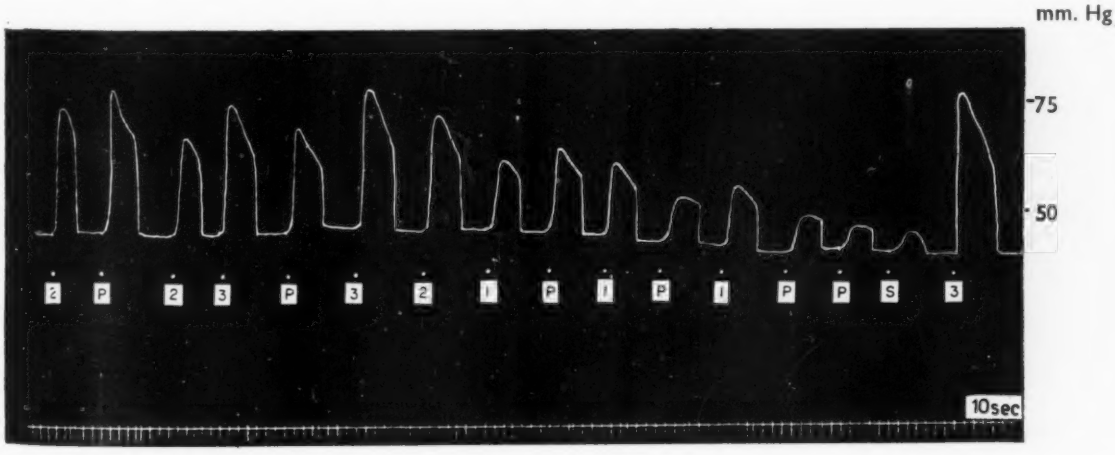

FIG. 1.—Arterial blood pressure of rat (245 g.). Urethane anaesthesia. Vagi cut and atropine given. 1, 2, and 3 = intravenous injections of 1, 2, and 3 μg. noradrenaline. P = 0.25 ml. (0.1 ml./100 g.) 20% solution of Witte's peptone. S = 0.25 ml. saline. The injections were made at 5 to 7 min. intervals.

the pressor response compared with that of noradrenaline. The rise in blood pressure in a group of 8 rats was comparable with that produced by the injection of 2 to 8 μg. of noradrenaline. In 3 rats a pressor response to a test dose of peptone was obtained. Both adrenal glands were then removed and 10 to 15 min. later the test dose of peptone was repeated. Fig. 2 shows the results obtained in one such experiment. Adrenalectomy apparently did not abolish or even reduce the magnitude of the pressor response produced by peptone which cannot therefore be due to the release of sympathin from the adrenal glands.

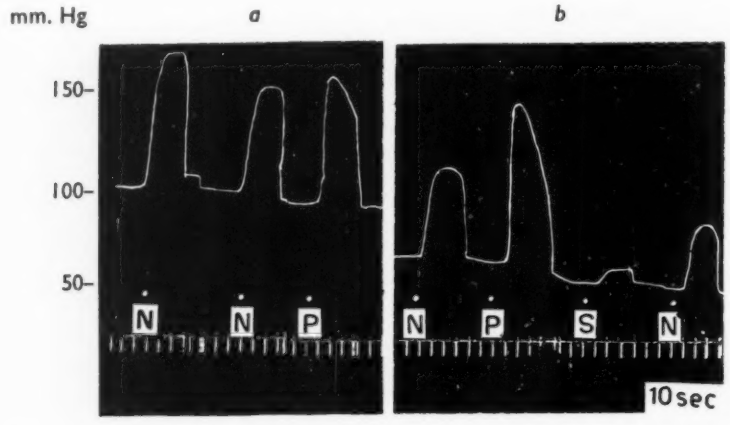

FIG. 2.—Arterial blood pressure of rat (300 g.). Urethane anaesthesia. Vagi cut and atropine given. N = intravenous injections of 2 μg. noradrenaline. P = 0.3 ml. (0.1 ml./100 g.) 20% solution Witte's peptone. S = 0.3 ml. saline. A period of 15 min. elapsed between (*a*) and (*b*) during which time both adrenals were removed. Before and after adrenalectomy the injections were made at 5 min. intervals.

Drugs Antagonizing the Peptone Pressor Effect.—It is known from the work of Bhattacharya and Lewis (1956) that histamine liberators (48/80, morphine and propamidine) release not only histamine but 5-hydroxytryptamine as well from the perfused hindquarter of rats. If this was the case with peptone, the liberation of 5-hydroxytryptamine might partially or completely account for the pressor effect following the injection of peptone in the rat. In 3 experiments in which the pressor effect of 5-hydroxytryptamine was completely reversed when the animals were treated previously with lysergic acid butanolamide tartrate, a potent inhibitor of 5-hydroxytyptamine, the pressor response to peptone still remained unchanged. Fig. 3 shows the results obtained in one of these experiments.

On the other hand, the pressor effect of peptone was antagonized by anti-adrenaline drugs such as ergotamine, dihydroergotamine, tolazoline, and dibenamine. The results obtained from 12 rats treated with one or other of these blocking agents showed that all of them were effective in reducing or blocking completely the pressor response to peptone. Fig. 4 shows the inhibition of the pressor response to peptone and noradrenaline by dihydroergotamine, under conditions in which

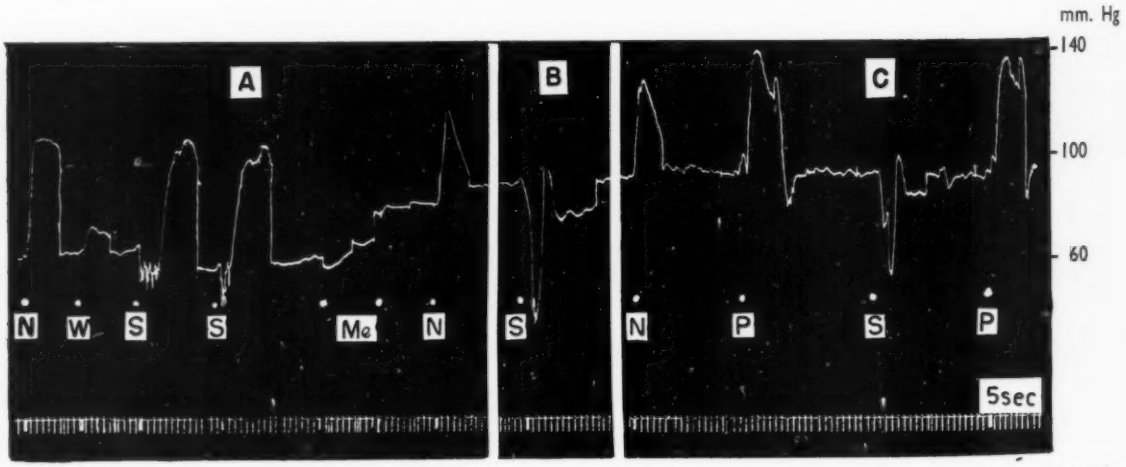

FIG. 3.—Arterial blood pressure of rat (235 g.). Urethane anaesthesia. N = intravenous injections of 2 μg. noradrenaline. W = 0.2 ml. saline. S = 40 μg. 5-hydroxytryptamine. Me = 200 μg. (+)-lysergic acid butanolamide (Methergin) on four repeated injections of 50 μg. each at 2 min. intervals between the two points marked in the recording. P = 0.12 ml. (0.05 ml./100 g.) 10% solution of peptone (Parke, Davis). A period of 10 min. elapsed between (A) and (B) and 8 min. between (B) and (C). The animal showed desensitization after the fifth injection of peptone. There was an interval of 5 min. between the injections.

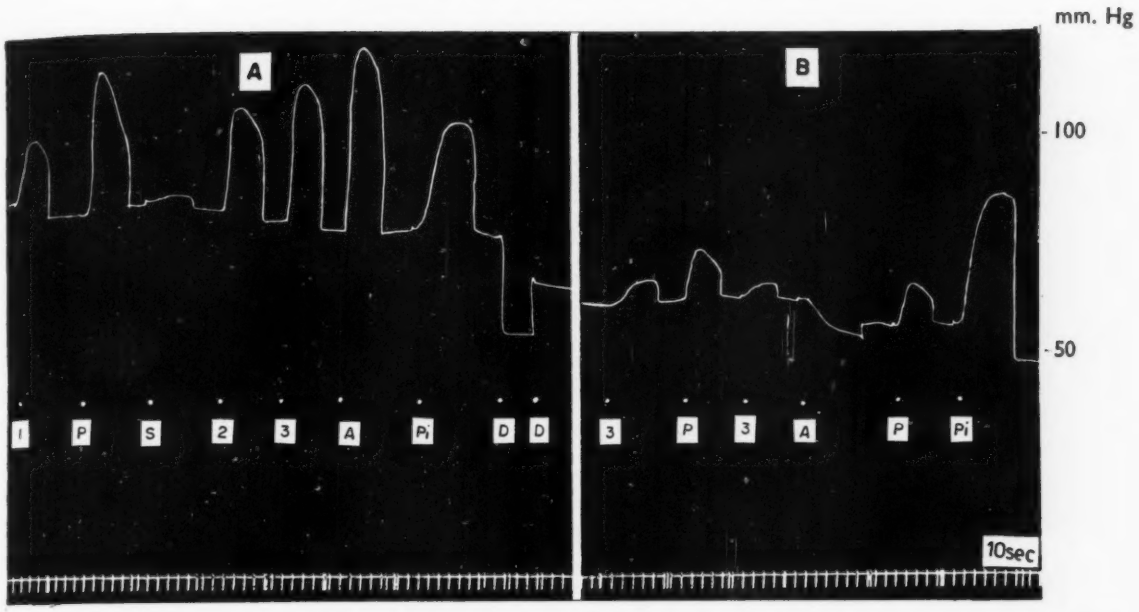

FIG. 4.—Arterial blood pressure of rat (235 g.). Urethane anaesthesia. Vagi cut and atropine given. 1, 2, and 3=intravenous injections of 1, 2, and 3 μg. noradrenaline. P=0.24 ml. (0.1 ml./100 g.) 20% solution Witte's peptone. S=0.25 ml. saline. A=3 μg. adrenaline. Pi=0.015 unit Pituitrin. D=500 μg. dihydroergotamine. A period of 3 min. elapsed between the two injections of dihydroergotamine and of 8 min. between (A) and (B). The injections were made at 5 min. intervals.

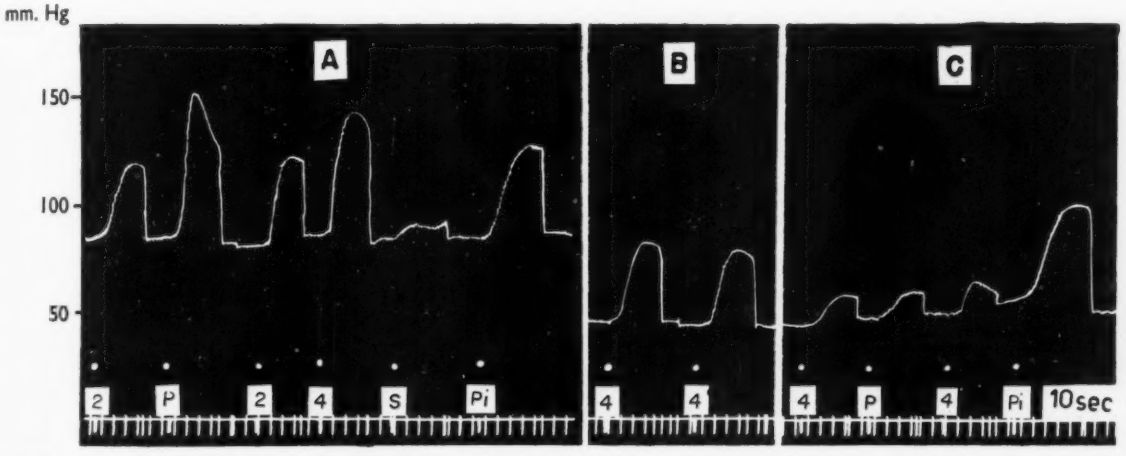

FIG. 5.—Arterial blood pressure of a spinal rat (330 g.). Urethane anaesthesia. Vagi cut and atropine given. 2 and 4=intravenous injections of 2 and 4 μg. noradrenaline. P=0.33 ml. (0.1 ml./100 g.) 20% solution Witte's peptone. S=0.4 ml. saline. Pi=0.01 unit Pituitrin. 5 mg. dibenamine was injected intravenously between (A) and (B) and between (B) and (C). A period of 27 and 40 min. elapsed between (A) and (B) and (B) and (C), respectively. Before and after dibenamine the injections were made at 5 min. intervals.

the pressor response to adrenaline was reversed. From the results of these experiments, it may be suggested that the pressor response to peptone is probably due to the release into circulation of noradrenaline or a noradrenaline-like substance.

Fig. 5 shows the complete blockade of peptone pressor effect by dibenamine in a spinal rat. The purpose of the experiment was to eliminate any influence of the higher nervous centres on the pressor response to peptone.

Nicotine and Hexamethonium.—After a series of intravenous injections of increasing doses of nicotine (50 μg. to 2 mg.), the last of which failed to cause a pressor response, the injection of peptone did not elicit a rise in blood pressure (5 experiments). Fig. 6 shows the results obtained in one such experiment.

In spite of the fact that, in a few experiments, hexamethonium potentiated the pressor response to exogenous noradrenaline it appeared to abolish

completely the pressor response to peptone (8 experiments). Partial recovery of the rise was observed 60 min. later, as shown in Fig. 7.

In order to test the effectiveness of ganglionic blockade after paralysing doses of nicotine and hexamethonium, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), a potent ganglion-stimulating drug studied by Chen, Portman, and Wickel (1951), was used. As both the blockade and recovery of the pressor effect of peptone and DMPP after nicotine and hexamethonium follow a parallel course as shown in Figs. 6 and 7, it is suggested that the effect of peptone in raising the blood pressure is due to stimulation of sympathetic ganglia. The gradual return of reactivity to peptone and ganglion-stimulating agent (DMPP) after the blockade induced by hexamethonium can easily be understood on the basis of the gradual recovery of the sensitivity of the ganglia as the inhibition dies out. The blockade produced by nicotine, on the other hand, remained for the rest of the experiment (90 min.).

Effect of Intravenous Injections of 48/80 and Histamine on the Blood Pressure.—It has been shown recently that histamine stimulates the superior cervical ganglion of the cat (Trendelenburg, 1954, 1955). As it was possible that the histamine released by peptone might cause the stimulation of the sympathetic ganglia, we decided to ascertain whether histamine set free from the tissue by the injection of 48/80 or injection of exogenous histamine would be able to duplicate the rise in blood pressure caused by peptone.

In 6 experiments 48/80 was injected intravenously in doses ranging from 50 μg. to 2 mg. and histamine in doses ranging from 20 to 500 μg./100 g. of body weight (10 experiments). Neither 48/80 nor histamine had any pressor effect, but

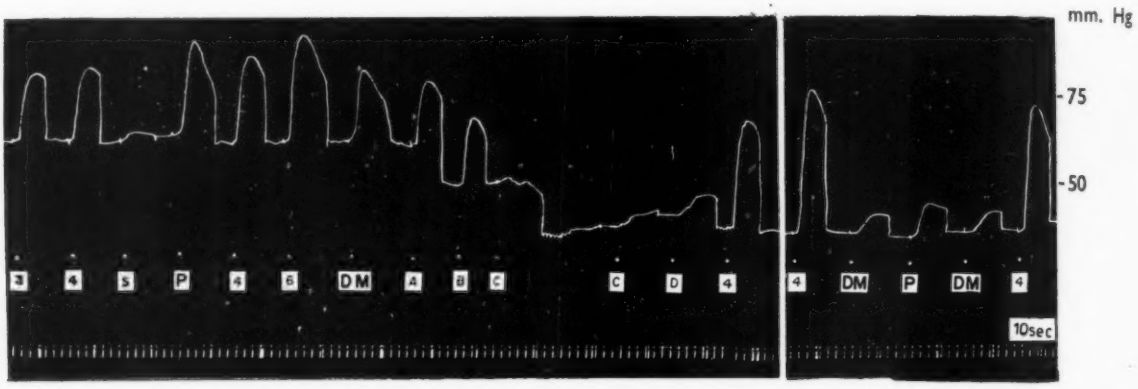

FIG. 6.—Arterial blood pressure of rat (316 g.). Urethane anaesthesia. Vagi cut and atropine given. Artificial respiration. 3, 4, and 6=intravenous injections of 3, 4, and 6 μg. noradrenaline. S=0.3 ml. saline. P=0.3 ml. (0.1 ml./100 g.) 20% solution Witte's peptone. DM=50 μg. 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP). Intravenous injections of increasing doses of nicotine 50 μg. (A), 100 μg. (B), 250 μg. (C) and 1 mg. (D). A second series of nicotine injections (10 mg. in all) was given between the two tracings. The injections were made at 5 to 7 min. intervals.

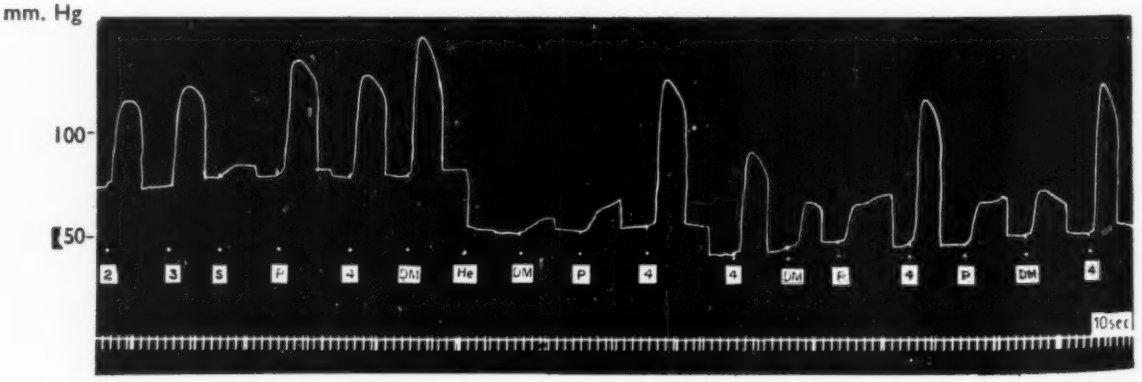

FIG. 7.—Arterial blood pressure of rat (364 g.). Urethane anaesthesia. Vagi cut and atropine given. Artificial respiration. 2, 3, and 4=intravenous injections of 2, 3, and 4 μg. noradrenaline. S=0.4 ml. saline. P=0.36 ml. (0.1 ml./100 g.) 20% solution Witte's peptone. DM=50 μg. DMPP. He=10 mg. hexamethonium. The injections were made at 5 to 7 min. intervals.

all 16 rats showed fall in the blood pressure in both cases. However, the animals showed typical responses to the injection of noradrenaline.

Discussion

The pressor effect of peptone is apparently a normal component of the action of this substance on the blood pressure of the rat. Since the pressor response was not abolished or diminished by adrenalectomy, the blood pressure increase could not have been caused by release of adrenaline or noradrenaline from the adrenal glands. Croxatto and Croxatto (1942) demonstrated that digestion of hypertensinogen (plasma globulin) and other proteins with pepsin leads to the formation of a pressor substance having pharmacological properties very similar if not identical with those of hypertensin (angiotonin). This substance was called pepsitensin. As it was possible that the presence of pepsitensin in peptone could be involved in the mechanism of the rise of blood pressure it is pertinent to emphasize the important pharmacological differences between the pressor response to pepsitensin and that obtained by the intravenous injection of peptone in the rat. According to Alonso, Croxatto and Croxatto (1943) repetition of intravenous injections of pepsitensin did not cause desensitization of the animal (tachyphylaxis), nor was the pressor response to pepsitensin influenced by adrenergic blocking drugs such as 2-piperidinomethylbenzo-1:4-dioxan (933F). On the other hand, desensitization could be observed after repeated injections of peptone and its pressor effect was abolished by adrenergic blocking drugs. In addition, the pressor response to peptone is blocked by paralysing doses of nicotine and hexamethonium. Although no reference to the action of ganglionic blocking agents on the response to pepsitensin could be found, Page and Taylor (1947) reported potentiation of the pressor response by hypertensin (a polypeptide considered homologous if not identical with pepsitensin) by tetraethylammonium. Finally, the peptone solution used throughout these experiments was previously treated with permutit in order to remove trace of histamine, and permutit also adsorbs pepsitensin from aqueous solutions (quoted by Paiva, 1954). Therefore, there is no indication that the pressor effect of peptone is due to the possible presence of pepsitensin in the peptone. The pressor effect also could have been caused by the release of 5-hydroxytryptamine, for it has been shown by Bhattacharya and Lewis (1956) that a series of histamine liberators is able to release 5-hydroxytryptamine from rat tissue. The pressor action of 5-hydroxytryptamine, however, was completely reversed by the butanolamide of lysergic acid (Methergin) under conditions in which the pressor effect of peptone remained unchanged.

Since it was not possible to duplicate the pressor response to peptone by either 48/80 or histamine, the hypothesis put forward that peptone releases sympathin through the histamine set free from the tissues can be ruled out.

The group of drugs which antagonizes or blocks the peptone-induced pressor response both in intact and in spinal rats suggests that the pressor effect may be due to a direct action of peptone on sympathetic ganglia. The antagonistic effect of the adrenergic blocking agents (dibenamine, ergotamine, dihydroergotamine and tolazoline) as that of ganglionic blocking drugs (nicotine and hexamethonium) would thus find an adequate explanation.

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A COMPARISON OF SOME ATROPINE-LIKE DRUGS IN MAN, WITH PARTICULAR REFERENCE TO THEIR END-ORGAN SPECIFICITY

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Atropine, methanthelinium, propantheline, oxyphenonium, hyoscine, and hyoscine methylbromide have been compared in man. After graded subcutaneous doses of these drugs simultaneous observations were made on the heart rate, salivary secretion, pupil size, near point of accommodation, micturition, and palmar sweating. This permitted a quantitative assessment of any differential effects of the drugs on the different end-organs.

Small doses which depressed salivary secretion and palmar sweating did not necessarily accelerate the heart or slow micturition. Atropine and hyoscine, which are tertiary amines, had a greater effect than the other (quaternary) drugs on the iris and the ciliary muscle, compared with the effects on the other end-organs studied. This difference may be related to the fact that quaternary compounds penetrate cellular membranes with difficulty.

The time course of the drug effects differed for different end-organs. The changes in heart rate, salivary secretion, and sweating began and ended sooner than those affecting accommodation and the pupil. As the dose of a drug was increased, the peak effect on the heart rate and salivary secretion tended to occur sooner, but the peak effect on the iris and ciliary muscle always occurred later. It is suggested that the aqueous humour may be acting as a reservoir for the drugs. After methanthelinium, all the drug effects began sooner and reached their peak sooner than after the other drugs.

The usefulness of atropine substitutes in man is largely determined by their end-organ specificity. If a drug paralyses all peripheral cholinergic effectors equally, it will inevitably produce more "side effects" than desired effects. To investigate whether drugs differ in the degree to which they affect different end organs, simultaneous observations have been made on several different responses in the same subject, after graded doses of the drugs. The drugs and doses used are shown in Table I.

TABLE I

DRUGS AND DOSES USED

The number of experiments is given in parentheses after each dose.

Drug	Doses in mg./70 kg.			
Atropine sulphate ..		0·5 (7)	1·0 (7)	2·0 (7)
Methanthelinium bromide	7·0 (4)	14·0 (2)	28·0 (5)	56·0 (4)
Propantheline bromide	2·1 (4)	4·2 (2)	8·4 (10)	16·8 (6)
Oxyphenonium bromide	0·5 (3)	1·0 (3)	2·0 (6)	4·0 (7)
Hyoscine methylbromide	0·125 (4)	0·25 (2)	0·5 (6)	1·0 (6)
,, hydrobromide		0·2 (3)	0·4 (4)	0·8 (3)

METHODS

The subjects were 12 normal men, between the ages of 22 and 35 years, and a man aged 50, convalescing from a gastric ulcer.

Each subject received two to four different doses of each of two or more drugs. Doses were related to the body weight of the subject before the first experiment. Successive experiments in the same subject were separated by at least three days. The subjects did not know what drug they were receiving on a particular occasion.

At the start of each experiment, baseline observations were made for ½ to 1 hr. with the subject lying on a couch. After this control period the drug was given by subcutaneous injection into the upper arm and observations were made at frequent intervals until the effects of the drug began to wear off. The subject remained recumbent until the heart rate had returned to within 10 beats/min. of its initial value.

Heart Rate.—Counts for ¼ to 1 min. were made at intervals of about 2 to 6 min. until it began to return to normal; thereafter the rate was counted less frequently. The increase in rate is expressed as % of the initial rate.

Salivary Secretion.—This was measured by a modification of the method of Mushin, Galloon and Lewis-Faning (1953). After the subject had swallowed all resting saliva, 0.2 ml. of 4% citric acid was dropped on the tongue, and held in the mouth for 30 sec. At the end of this time 4 ml. of water was taken into the mouth and kept there for 15 sec., then the contents of the mouth were voided into a measuring cylinder. This can be repeated every 15 to 20 min. The % inhibition of salivary secretion has been calculated from the expression: $100 \times (V_o - V_a)/V_o$, where V_o is the mean volume of saliva obtained before administration of the drug and V_a the volume obtained after giving the drug.

Pupil Diameter.—The diameter of the pupil of one eye was measured by a simple pin-hole pupillometer (Reid, 1955). In this indirect method the intensity of illumination of the eye is low, since the pupillometer keeps out most of the extraneous light. (Unpublished observations made after the completion of the experiments described in this paper have shown that changes in pupil size produced by an atropine-like drug are much more readily apparent when the pupil is measured in bright light.) Measurements to the nearest 0.25 mm. were made in triplicate at intervals of 20 to 30 min. The results have been expressed in terms of the increase in pupil diameter in mm.

Near Point.—The near point of accommodation of one eye was measured with a near point rule every 20 to 30 min.; the results have been expressed in dioptres. Each point represents the mean of three measurements. Subjects who normally wore spectacles wore them for these measurements. In order to make it possible to compare the degrees of paralysis of accommodation obtained in different subjects, the range of accommodation from the near point to the far point was determined for each subject. This was done on an occasion when no drug had been given. When the eye was hypermetropic, a positive lens was placed in front of the eye, such that a distant object was still just in focus. The dioptric power of this lens was added to the range of accommodation between the near point and infinity. In each subject, paralysis of accommodation has been expressed as % of this range: $\frac{D_o - D_m}{D_o + L} \times 100$, where D_o is the near point before the injection, D_m the near point when the action of the drug was at its peak, and L the dioptric power of the lens mentioned.

Sweating.—Sweating on the pad of one middle finger was assessed by an adaptation of the method described by Wada (1950) for the detection of sweating. The skin was painted with 3% iodine in 95% alcohol, and allowed to dry. A thin film of a 1:1 mixture of soluble starch and castor oil was then spread on the area so that each active sweat gland appeared as a discrete black dot. The time in seconds taken for an arbitrary number of black dots to become visible under a lens was taken as an index of sweat gland activity. (If, after a drug had been administered, sweat dots took twice as long to appear, this was taken to indicate 50% inhibition of sweating.)

Micturition.—The time taken to empty the bladder was recorded and the volume of urine measured. The quotient volume/time (ml./sec.), referred to as the "micturition speed" (S_m), has been used as a measure of the effect of the drugs on micturition. To minimize psychological interference, the subject went into a special lavatory, and timed each micturition himself, using a stop-watch. Since it was necessary to leave the couch for this purpose, micturition was only encouraged after the heart rate had passed its peak. In some experiments the subject drank suitable quantities of water beginning about 1 hr. after the drug had been given, in order to enable more frequent observations to be made. This does not affect the volume of saliva secreted (de Wardener and Herxheimer, 1957), nor was there any evidence that it affected the heart rate. Since the S_m varies with the volume passed (Herxheimer, unpublished observations), each S_m obtained under the influence of a drug has been compared with the normal S_m for a similar volume of urine in the same subject. Even when similar volumes are passed on different occasions, however, the speeds may differ considerably, and this limits the accuracy with which it is possible to determine the decrease in S_m attributable to a drug. In the present experiments further errors were introduced, for no observations were made until the heart rate had passed its peak; since the rate of urine flow varied on different occasions, micturition was not always possible at comparable times after injection of the drug. This error due to difficulties in timing affected all the drugs to about the same extent. In many experiments only one or two observations were made in the first 3 hr., so that the peak effect on the bladder is likely to have escaped observation. The % decrease in S_m is given by the expression: $100 \times S_m$ (after drug)/S_m (control). When more than one control observation was available for a given volume, the lowest control S_m was used in the calculation of % decrease.

Other Effects.—These were not specifically looked for, but subjects were asked to report any unusual sensation; direct questions were not asked in this connexion. Obvious changes (such as sleep, flushing of the face) were noted by the observer.

Statistical Procedures.—Log dose/effect regression lines were calculated from the maximum effects of the different doses on the heart rate, salivary secretion, pupil diameter and accommodation. The regressions were tested for linearity and parallelism, and in the absence of a significant departure from parallelism potency ratios were calculated using a common slope. Analyses of variance were carried out to allow for differences between subjects. For this purpose it was necessary to calculate the missing values in each group of observations, and equations of the form described by Finney (1950) were solved by successive approximation. The residual errors obtained from these

analyses of variance were used in testing the significance of differences between any two regression lines.

The results on the time course of the drug effects were examined by analyses of variance, by testing the significance of correlation coefficients, or by *t* tests. Differences between subjects were not allowed for except in the results on accommodation. The treatment of these results is described below.

The coefficient of precision (λ) was calculated, for each of five responses, from the pooled residual error variance of all the observations used in the calculation of regression lines, and from the common slope.

Results

A typical experiment is illustrated in Fig. 1. The extent of the changes, and their time course, can be seen for each effect investigated.

Dose/Effect Relationships

Heart Rate.—All drugs except hyoscine caused an increase in the heart rate (Fig. 2*a*). Hyoscine either produced a fall or no change and is therefore not shown in Fig. 2*a*. Four of the five log dose/effect lines do not deviate significantly from linearity if doses giving less than a 20% increase in heart rate are excluded. The response to propantheline is linearly related to the log dose except at the highest dose, which has therefore not been used in the calculation of potency ratios. The regression line for atropine is steeper than the lines for the other drugs, but the difference in slope is not significant at the 5% level ($0.1>P>0.05$). A common slope was therefore used to calculate the potency ratios in column (a) of Table II.

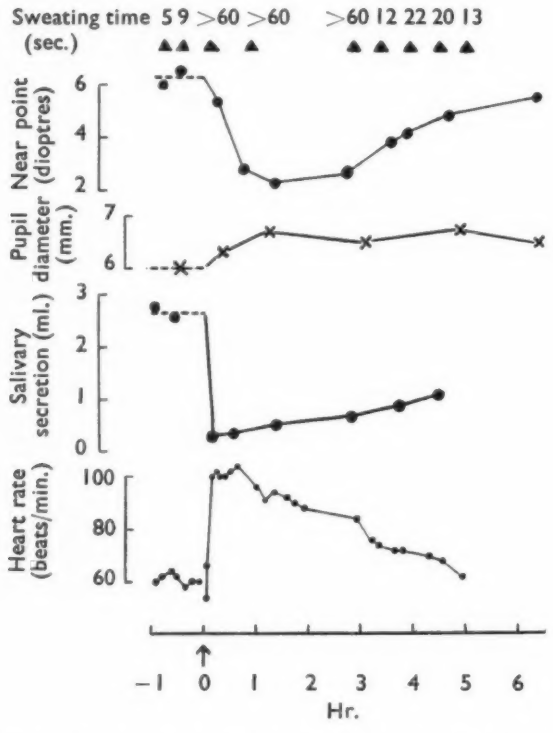

FIG. 1.—The changes observed in one experiment following subcutaneous injection of 56 mg./kg. of methanthelinium bromide at zero time. 2 hr. after the injection the subject voided 93 ml. urine at an average speed of 2 ml./sec.; at 4 hr., 512 ml. at a speed of 9 ml./sec.; and at 6½ hr., 293. ml at a speed of 11 ml./sec

TABLE II

RELATIVE POTENCIES OF ATROPINE-LIKE DRUGS

The potencies have been calculated in terms of mols of the bases. Atropine has been given an arbitrary value of 1.0.

Drug	(a) Acceleration of Heart Rate	(b) Inhibition of Salivary Secretion	(c) Slowing of Micturition	(d) Dilatation of Pupil	(e) Paralysis of Accommodation	(f) Ratio (b)/(e)
Atropine	1·0	1·0	1·0	1·0	1·0	1·0
Methanthelinium ..	0·20	0·22	0·26	0·06	0·11	1·9
Propantheline ..	0·70	0·76	0·59	0·08	0·22	3·5
Oxyphenonium ..	1·4	1·7	0·96	0·29	0·53	3·2
Hyoscine methylbromide	5·6	6·2	2·4	3·2	1·7	3·7
Hyoscine hydrobromide	—	3·7	2·4	5·1	6·5	0·56

Salivary Secretion.—All the drugs strongly inhibited salivary secretion in the doses used (Fig. 2*b*). Only two points for each of four drugs were sufficiently low on the log dose/effect curves to allow the calculation of potency ratios. All the points for methanthelinium and propantheline, and the remaining points for the other drugs, appeared to lie on the upper non-linear portion of the log dose/effect curve. The common slope was therefore calculated from the regression lines for the two lowest points of atropine, hyoscine, hyoscine methylbromide, and oxyphenonium, which are parallel. The potencies of methanthelinium and propantheline in Table II have been estimated on the assumption that log dose/effect lines parallel with the others pass through the lowest point obtained with each drug. The differences in potency shown in Fig. 2(*b*) between oxyphenonium and atropine, and methylhyoscine and hyoscine, are significant at the 5% level.

Pupil Diameter.—Only doses which caused a mean increase in the pupil diameter of more than 0.3 mm. were used in the calculation of the log dose/effect lines (Fig. 3*a*). The relative potencies of the drugs are given in Table II. It is noteworthy that atropine is more potent than oxyphenonium ($P<0.05$), and that hyoscine is about as potent as its methyl derivative.

Accommodation.—The paralysis of accommodation varied greatly between subjects. Two subjects who were myopes showed weak and irregular responses, and were not included. Only doses producing a mean paralysis greater than 7% of the whole range of accommodation were used to calculate log dose/effect lines (Fig. 3*b*). All but the two highest doses of all the drugs except methanthelinium were thus excluded. The three subjects with hypermetropia gave consistently bigger responses than the emmetropes; this probably explains why the standard errors in Fig. 5 are so large. Since each point is the mean of observations on varying numbers of hypermetropes and emmetropes the slope of the log dose/effect lines is falsified unless allowance is made for this heterogeneity. The means have therefore been adjusted so that equal weight is given to the results obtained from hypermetropes and emmetropes. This adjustment was disregarded in computing standard errors and precision coefficients. It appears (Table II, Fig. 3*b*) that hyoscine is more potent than its methyl derivative, and atropine than oxyphenonium, but the differences do not reach the 5% level of significance.

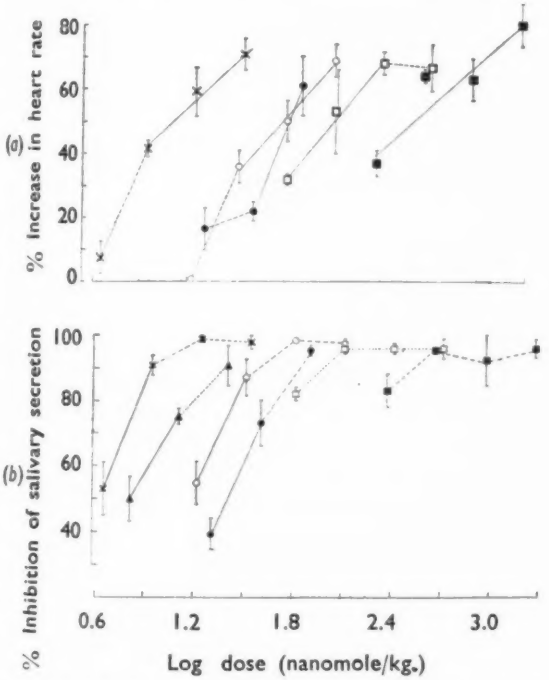

FIG. 2.—Log dose/effect curves for heart rate (*a*) and salivary secretion (*b*). The full lines are calculated regression lines; the broken lines join points lying on the non-linear portions of the dose/effect curves. The standard error of the mean is given for each point. ×, hyoscine methylbromide; ▲, hyoscine hydrobromide; ○, oxyphenonium; ●, atropine; □, propantheline; ■, methanthelinium.

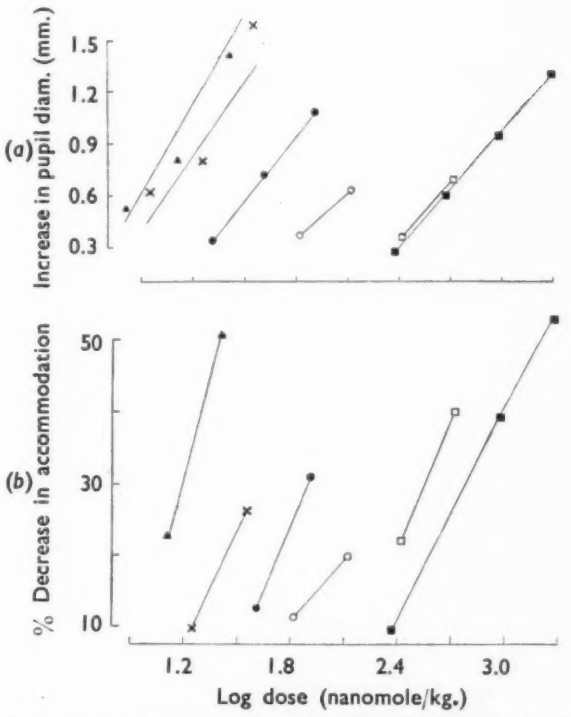

FIG. 3.—(*a*) Calculated log dose/effect regression lines for pupil diameter. (*b*) Log dose/effect lines for accommodation. The points for accommodation are weighted means (see text). The standard errors of the means shown amounted to between 20% and 50% of the means, the average being 35% for the observations on pupil diameter, and 28% for those on accommodation. ×, hyoscine methylbromide; ▲, hyoscine hydrobromide; ○, oxyphenonium; ●, atropine; □, propantheline; ■, methanthelinium.

Sweating.—Three of the subjects normally showed only minimal sweating on the palms and finger pads, and were therefore excluded. In the others, nearly all the doses inhibited sweating on the finger pad by more than 85%. The lowest dose of atropine (0.5 mg./70 kg.) was the only one that caused less inhibition (mean 65%). It was therefore not possible to estimate potency ratios for this response.

Micturition.—The results are given in Fig. 4. Hyoscine and methylhyoscine were approximately equipotent, and so were atropine and oxyphenonium. The potency ratios in Table II have been calculated from the doses causing a 50% decrease in S_m. These were obtained by graphical interpolation. Methanthelinium is the only drug which appeared to be relatively more potent in its action on micturition than on the other responses.

Other Effects.—All the doses of hyoscine used produced drowsiness, and in many experiments the subjects felt giddy and confused. Drowsiness was also observed after the higher doses of the

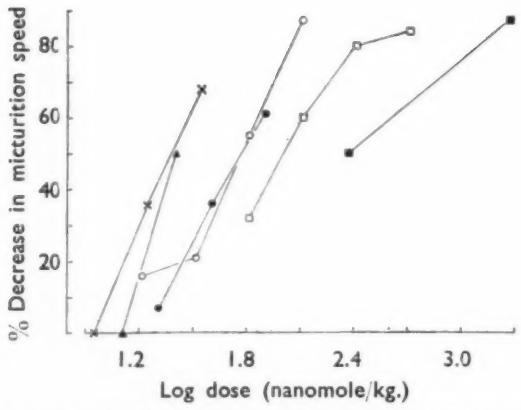

FIG. 4.—Log dose/effect curves for micturition speed. The standard errors of the means for the points, reading from left to right for each drug, are given in parentheses. ×, hyoscine methylbromide (0, 24, 12); ▲, hyoscine hydrobromide (0, 17); ○, oxyphenonium (9, 12, 28, 5); ●, atropine, (5, 8, 7); □, propantheline (7, 3, 7, 6); ■, methanthelinium (18, 9).

other drugs. The incidence was greatest after methanthelinium and least after atropine (Table III).

Flushing of the face occurred in over half the experiments, and was often accompanied by a sensation of warmth. This effect usually occurred 15 to 20 minutes after the injection.

Uncomfortable dryness of the nose and eyes was noted in a number of experiments. Other symptoms that were occasionally reported included mild headache and epigastric discomfort. Constipation on the following day occurred after three experiments with propantheline and oxyphenonium. In one subject, extra-systoles were observed in 5 experiments, 3 with propantheline and 2 with oxyphenonium.

TABLE III

INCIDENCE OF DROWSINESS

The doses are expressed as multiples of the lowest dose used (see Table I). The proportion of experiments in which the subject was drowsy or fell asleep is given for each dose.

Drug	Dose			
	1	2	4	8
Atropine	1/7	2/7	0/7	
Methanthelinium	0/4	0/2	2/5	4/4
Propantheline	1/4	0/2	2/10	4/6
Oxyphenonium	0/3	0/3	3/6	2/7
Hyoscine methylbromide ..	2/4	0/2	1/6	2/6
,, hydrobromide ..	3/3	4/4	3/3	

Accuracy of Measurements

In order to obtain an estimate of the precision of the methods used, precision coefficients (λ) were calculated from the results on heart rate, salivary secretion, pupil diameter, accommodation, and micturition. The coefficients were: heart rate, 0.187 (range for individual regressions, 0.131 to 0.212); salivary secretion, 0.073 (range, 0.015 to 0.119); pupil diameter, 0.466 (range, 0.315 to 0.570); accommodation, 0.171 (range, 0.098 to 0.276); micturition speed, 0.214 (range, 0.054 to 0.290). The coefficient is thus greatest for the measurements of the pupil, and smallest for those of salivary secretion.

Time Course of Drug Effects

Information on the time course of drug effects was obtained for all the responses studied, but since the heart rate was recorded at 3 to 6 min. intervals, and the other responses only at intervals of 15 min. or longer, the observations on the heart rate reflect the time course more precisely than do the others. The changes in the less frequently recorded responses can thus be expected to show up only major differences between drugs and between organs.

Heart Rate

Onset of Effect.—The intervals after which the heart rate began to increase are illustrated in Fig. 5. After atropine, methylhyoscine, oxyphenonium, and propantheline, the interval shortened significantly as the dose increased ($P<0.05$); there was no difference in the case of methanthelinium. Fig. 7 can be used to compare the times of onset of the effect following equi-active doses of the drugs. Table IV gives the results of such a comparison for doses causing a 50% increase in the heart rate. These doses have been derived from the individual log dose/effect regression lines, because the slope for atropine differed from the others, so that the use of the common slope might have caused some distortion. The heart rate began to rise much sooner after methanthelinium than after the other drugs.

TABLE IV

MEAN TIMES OF ONSET AND OF PEAK EFFECT, AND DURATION OF EFFECT ON THE HEART RATE AFTER ADMINISTRATION OF EQUI-ACTIVE DOSES CAUSING A 50% INCREASE IN HEART RATE

The equi-active doses have been calculated from the individua regression lines for the drugs; they are expressed in terms of the salts. See text for explanation of duration.

	Dose (mg./ 70 kg.)	Time of Onset (min.)	Time of Peak Effect (min.)	Duration (hr.)
Atropine	1·6	19	37	1·2
Methanthelinium	12·0	4	20	1·5
Propantheline	4·1	14	34	2·3
Oxyphenonium	1·9	16	37	2·4
Hyoscine methylbromide ..	0·36	18	40	2·3

Peak Effect.—Fig. 5 also shows the times at which the maximum effect on the heart rate was observed. In the case of atropine the peak occurred significantly sooner after the higher dose ($P<0.05$); methylhyoscine appeared to behave similarly ($P<0.1$). The times of the peak effects after oxyphenonium, propantheline, and methanthelinium were independent of the doses used. When equi-active doses are compared (Table IV) the peak occurred much sooner after methanthelinium than after the other drugs.

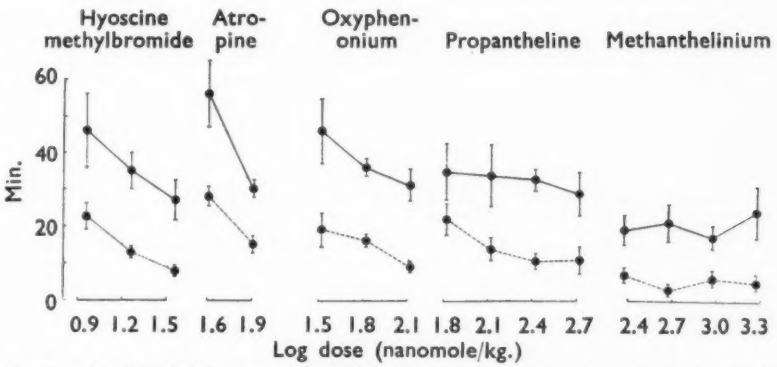

FIG. 5.—The effect of dose on the time at which the heart rate began to increase (broken line) and on the time of the maximum effect (full line). The standard error of the mean is given for each point.

Duration of Effect.—The duration of the effect on the heart rate was arbitrarily taken as the time during which the heart rate exceeded the pre-injection rate by 10 beats/min. or more. Fig 6*a* shows log dose/duration curves for the five drugs. There is no significant deviation from linearity, and the slopes are parallel. The duration of the effect following equi-active doses of the drugs is given in Table IV; it was shorter for atropine and methanthelinium than for the other three drugs.

Salivary Secretion

The time of onset of the effect on salivary secretion was not determined precisely, but the times between which inhibition began are known. In 30 out of the 36 experiments with methanthelinium and propantheline, inhibition was observed within 9 min. of the injection. (In the other 6 experiments salivary secretion was not tested in the first 10 to 20 min.) In most of the experiments with the other drugs inhibition had not begun 5 min. after the injection, and was only detected after 20 to 30 min., when the second observation on salivary secretion was made. Table V gives the approximate time of onset of inhibition after equi-active doses of the drugs. These were derived from the regression lines using the common slope.

The times at which the inhibition was maximal are shown in Fig. 7. In the experiments with atropine, hyoscine, hyoscine methylbromide, and oxyphenonium the peak occurred earlier as the dose was increased ($P<0.02$). After propantheline and methanthelinium the time of the peak effect was not related to the dose. In the experiments with methanthelinium the peak was reached much more rapidly than after any of the other drugs. This is exemplified in Table V.

The time during which salivary secretion was inhibited more than 50% was linearly related to the log dose for all the drugs (Fig. 6*b*). The log

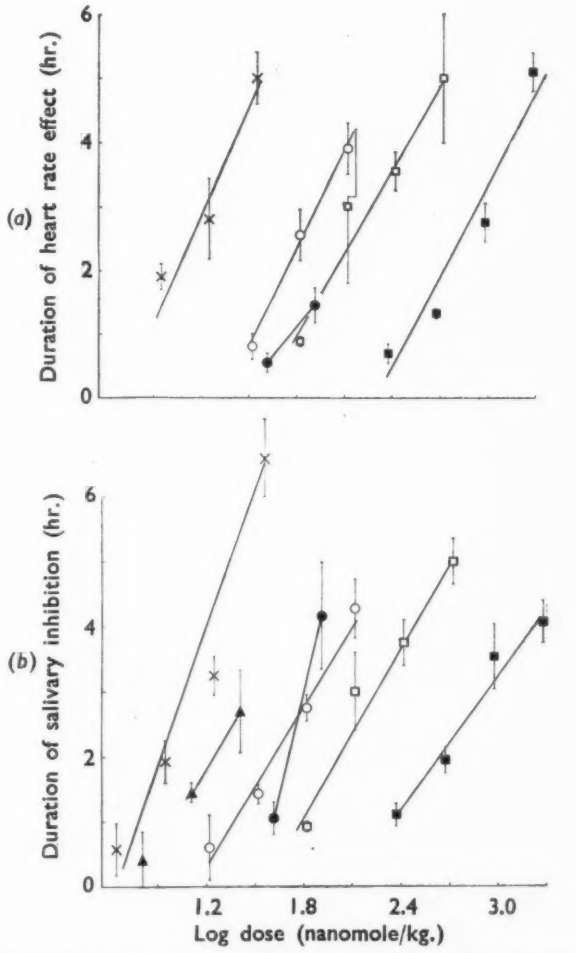

FIG. 6.—Calculated log dose/duration regression lines for the effects on heart rate (*a*) and salivary secretion (*b*). The duration of the effect on the heart rate has been arbitrarily taken to be the period during which it exceeded the resting level by 10 or more beats/min. The duration of the effect on salivary secretion was the period during which it was inhibited by 50% or more. The standard error of the mean is given for each point. ×, hyoscine methylbromide; ▲, hyoscine hydrobromide; ○, oxyphenonium; ●, atropine; □, propantheline; ■, methanthelinium.

TABLE V

MEAN TIMES OF ONSET AND OF PEAK EFFECT, AND MEAN DURATION OF EFFECT ON SALIVARY SECRETION AFTER ADMINISTRATION OF EQUI-ACTIVE DOSES CAUSING 85% INHIBITION OF SALIVARY SECRETION

Doses are expressed in terms of the salts.

Drug	Dose (mg./70 kg.)	Time of Onset (min.)	Time of Peak Effect (min.)	Duration (hr.)
Atropine	1·2	>7	37	2·1
Methanthelinium	7·1	<4	14	1·1
Propantheline	2·2	<5	25	1·2
Oxyphenonium	0·95	>6	34	1·6
Hyoscine methylbromide ..	0·23	>9	54	1·9
,, hydrobromide ..	0·52	>7	39	1·9

dose/duration lines for atropine and methylhyoscine are steeper than those for the other drugs ($P<0.01$).

Pupil Diameter

The effect on the pupil began later than that on the heart rate and salivary secretion, and took much longer to reach its peak. The peak effect occurred later after high doses than after low ones (Table VI).

TABLE VI

MEAN TIME OF MAXIMUM EFFECT ON PUPIL

These means are not strictly comparable with those shown in Fig. 3 (*a*) since experiments in which the pupil was not affected could not contribute to the means in this table. Single observations are marked with an asterisk. The doses refer to the weights of the salts.

Drug	Dose (mg./70 kg.)	Mean Time of Maximum Effect on Pupil Hr. After Injection ± Standard Error of Mean
Atropine	0·5	1·5±0·5
	1·0	4·1±1·2
	2·0	3·3±0·6
Methanthelinium	14·0	1·1*
	28·0	2·5±0·6
	56·0	2·4±0·4
Propantheline	8·4	1·8±0·1
	16·8	5·2±1·4
Oxyphenonium	2·0	1·5±0·2
	4·0	5·5±2·7
Hyoscine methylbromide ..	0·25	0·6*
	0·5	2·5±0·8
	1·0	4·1±0·8
,, hydrobromide ..	0·2	1·8±0·1
	0·4	1·6±0·4
	0·8	7·5±1·5

The duration of the effect was taken to be the time during which the pupil diameter exceeded the control diameter by 0.5 mm. or more. On several occasions, after high doses had been given, the pupil was still enlarged at the end of the experiment; in these cases "mean duration" was a minimum rather than a true mean (Table VII).

Accommodation

Changes in the near point began at variable times after injection of the drug. The delay was greatest with atropine, and least with methanthelinium. The timing of the peak effect was significantly related to the dose, in the experiments with atropine, propantheline and methanthelinium ($P<0.05$): the higher the dose, the longer the interval between the injection and the peak effect (Fig. 7). A similar trend was apparent with the other drugs. The duration of the effect was taken to be the time during which the near point of accommodation was decreased by 0.5 dioptre or more. A weighted mean duration was calculated as described above for each dose, so that observations from emmetropes and hypermetropes contributed equally. The results are given in Table VII.

TABLE VII

MEAN DURATION OF EFFECTS ON THE EYE

The duration of the effect on accommodation was taken to be the time during which voluntary accommodation was diminished by 0·5 dioptres or more. The means have been weighted as described in the text. The duration of the effect on the pupil was taken to be the time during which the pupil diameter exceeded the control diameter by 0·5 mm. or more. Each mean is followed by its standard error. The value marked with an asterisk was based on a single observation.

Doses are expressed in terms of the salts.

Drug	Dose (mg./70 kg.)	Mean Duration of Effect hr. (±Standard Error of Mean)	
		Accommodation	Pupil
Atropine	0·5	0·4 (±0·2)	0·1 (±0·1)
	1·0	2·6 (±1·2)	3·8 (±1·7)
	2·0	8·8 (±3·4)	7·3 (±1·5)
Methanthelinium	7·0	0·6 (±0·4)	0
	14·0	2·1*	1·5 (±1·5)
	28·0	6·5 (±2·2)	4·6 (±2·2)
	56·0	8·5 (±0·3)	7·1 (±1·0)
Propantheline	8·4	5·0 (±1·2)	0·3 (±0·3)
	16·8	13·9 (±2·4)	2·2 (±2·2)
Oxyphenonium	2·0	2·0 (±1·4)	0·4 (±0·3)
	4·0	3·8 (±1·7)	1·4 (±1·3)
Hyoscine methylbromide ..	0·5	0·6 (±0·2)	0·7 (±0·3)
	1·0	11·8 (±3·0)	4·8 (±2·2)
,, hydrobromide ..	0·2	0·1 (±0·1)	1·2 (±0·9)
	0·4	3·2 (±1·1)	2·8 (±1·2)
	0·8	10·2 (±1·2)	4·7 (±2·6)

Sweating

In most experiments sweating began to diminish within 30 min. of the injection, and the peak effect usually occurred between 30 and 60 min. after the injection. After methanthelinium, the onset was more rapid than after the other drugs (mean, 13 min.), and the peak came sooner (mean, 17 min.). There was no consistent relation between the time of the peak effect and the dose.

The duration of 50% inhibition of palmar sweating, estimated by interpolation, was of the same order as the duration of 50% inhibition of salivary secretion. The slopes of the log dose/duration lines were similar to those shown for salivary secretion in Fig. 6*b*, except in the case of hyoscine. The two lowest doses of this drug

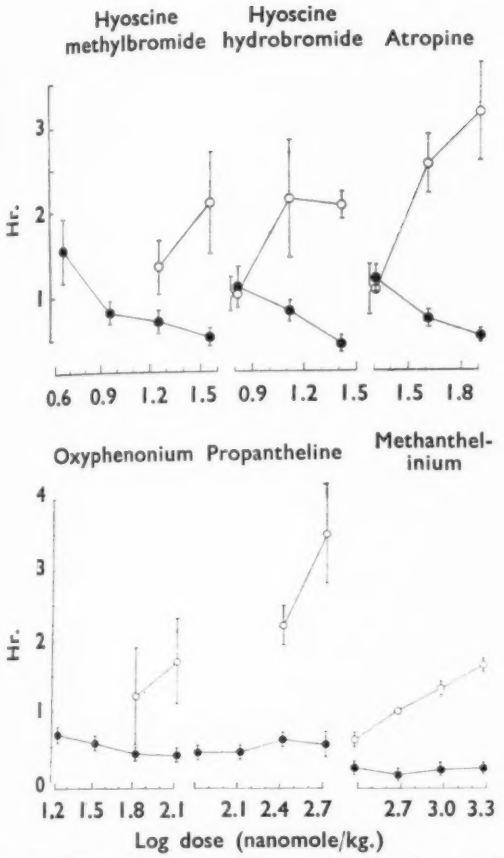

FIG. 7.—Effect of dose on the time of occurrence of the maximum effect on salivary secretion (●) and on accommodation (○). The standard error of the mean is given for each point.

inhibited sweating for rather longer than they inhibited salivary secretion, so that the slope was less steep.

Micturition

The micturition speed frequently remained below 50% of normal for over 5 hr. after the highest doses of the drugs. The results were insufficient for a detailed examination of the duration of this effect.

Initial Stimulant Effects

In a proportion of experiments various paradoxical stimulant effects occurred before the customary inhibition began. Cardiac slowing frequently preceded acceleration, salivary secretion was often increased when tested within 8 min. of the injection, in some experiments there was a transient increase in the power of accommodation, and in a few pupillary constriction preceded dilatation. These paradoxical results will be reported in detail elsewhere.

DISCUSSION

Some of the effects of the drugs on the heart rate were surprising. Hyoscine slowed the heart in most experiments, and never caused acceleration. Anaesthetists should therefore not rely on hyoscine if they wish to block the vagus. When the other drugs were given, acceleration of the heart was frequently preceded by slowing, indicating that these drugs have a dual action on the heart rate, and the dose/response relationship presumably results from the interaction of these two opposing effects. Compared with the quaternary drugs, atropine seems to produce relatively more slowing than acceleration, and it is possible that differences of this kind also exist between the various quaternary drugs. Such considerations might explain the difference in slope between the line for atropine and those for other drugs, and also the anomalous response to the highest dose of propantheline (Fig. 2*a*). Another, but perhaps less plausible, explanation for this response which is much smaller than one would expect might be steric hindrance at the receptors.

All the drugs had a much greater effect on salivary secretion than on the heart rate; this has long been known to be so in the case of atropine (Henderson, 1923). Palmar sweating also appeared to be very sensitive to all the drugs. Therapeutic doses of atropine-like drugs only infrequently cause overt disturbances of micturition, but the results show that they produce definite slowing in normal subjects. This suggests that post-operative retention of urine may on occasion be due to the premedication.

The potency ratios in Table II show a remarkably consistent pattern. The ratios for the effect on the heart rate and salivary secretion run parallel for each drug, and the same is true for the effects on the pupil and on accommodation. When the effect on salivary secretion is compared with those on accommodation and the pupil, however, a striking difference is found. The ratios given in column (f) of Table II are much lower for hyoscine and atropine, the tertiary amines, than for the quaternary compounds, indicating that the tertiary compounds gain access to the ciliary muscle and iris more easily than the quaternary ones; the order is hyoscine, atropine, methanthelinium, and then the other three drugs. This order resembles that of the relative potencies of the drugs in causing drowsiness in the present experiments. Hyoscine was much more active than the quaternary drugs, and of these methanthelinium appeared to be a little more potent than the others. Atropine was least active, presum-

ably because it causes central stimulation rather than depression.

The relative potencies of the drugs in slowing micturition were not strikingly different from the figures for salivary secretion and heart rate, despite the possibility that an effect on the bladder may in part be due to the presence of drug in the urine. Levine and Clark (1955) have shown that, after intravenous infusion of methanthelinium in the dog, nearly 30% of the drug is excreted in the urine within 2 hr. If this also occurs in man, it might explain why methanthelinium is relatively more potent in its effect on micturition than in its action on salivary secretion and heart rate.

The time course of the drug effects shows a number of interesting features. With one exception there was little difference between drugs in the length of the latent period before the onset of the various effects. The exception was methanthelinium, whose actions became manifest much earlier than those of the other drugs. This suggests that it may be more rapidly absorbed from the site of injection than the other drugs, but this finding could also be explained in other ways.

The time course was not the same for the different effects studied; paralysis of accommodation and dilatation of the pupil mostly began later and lasted longer than the effects on the heart rate and salivary secretion. The maximum effects on accommodation and pupil also occurred later than those on the heart rate and salivary secretion. The higher the dose of a particular drug, the later did the peak effects on the eye occur (Fig. 7). The peak effects on the heart rate and salivary secretion behaved quite differently: they tended to occur earlier as the dose was increased. All these findings imply that the effects of the drug on the eye were still increasing although the concentration of the drug at other sites had been falling for some time. This suggests the existence of some reservoir which accumulates the drug while its concentration in the extracellular fluid is high, and then slowly gives it off to the iris and ciliary muscle. The aqueous humour might plausibly act as such a reservoir, although it seems at first sight surprising that the ciliary muscle and iris, which are well supplied with vessels, should take up much greater amounts of all the drugs from the aqueous humour than from the blood.

Involvement of the aqueous in the passage of drug from the blood to the iris and ciliary muscle could also explain why the quaternary compounds are less potent in their actions on the eye than the tertiary amines atropine and hyoscine. The blood-aqueous humour barrier has many features in common with the blood-cerebrospinal fluid barrier (Davson, 1956), and there is evidence that quaternary ammonium compounds pass into the cerebrospinal fluid much less readily than do tertiary amines (Paton and Zaimis, 1952; Bhattacharya and Feldberg, 1958). It has also been noted that many quaternary ammonium compounds are much less potent in their action on the central nervous system than closely related tertiary compounds (Nyman, 1943; Ing, Dawes, and Wajda, 1945).

This work would not have been begun without the enthusiasm and help of Dr. Cyril Sanger. I am very grateful to him and to the subjects, to Dr. J. O. Irwin for help with the statistics, and to Dr. R. S. Stacey and other colleagues for their interest and criticisms. I am indebted to G. D. Searle and Co., Ciba Laboratories Ltd., and Upjohn of England Ltd. for generous supplies of drugs. In addition some of the expenses of the work were defrayed by G. D. Searle & Co. and Ciba Laboratories Ltd.

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THE EFFECT OF ANTICHOLINESTERASES ON THE PAROTID GLAND AFTER PARASYMPATHETIC DECENTRALIZATION OR DENERVATION

BY

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Anticholinesterases (eserine, ethyl pyrophosphate, paraoxon) were injected into the parotid ducts and found to cause a secretion of saliva. After previous preganglionic, parasympathetic denervation the effects were increased above normal; this was probably due to the supersensitivity to acetylcholine which develops after the operation. After previous postganglionic, parasympathetic denervation, on the other hand, the effects were much reduced, in spite of a pronounced supersensitivity towards, for example, acetylcholine. The cause of this "subsensitivity" towards cholinesterase inhibitors is discussed.

In a recent monograph Burn (1956) has quoted several investigations demonstrating, as apparent exceptions of Cannon's law of denervation, that denervated structures may show a diminished responsiveness, or none at all, towards certain chemical agents which stimulate the normally innervated structures. Anderson (1905) found, for instance, in his classical experiments on the pupil that eserine some time after excision of the ciliary ganglion caused no miosis, whereas pilocarpine had a greater effect than normally.

After previous section of the chorda tympani, eserine still has a pronounced secretory effect on the submaxillary gland (Dirnhuber and Evans, 1954; Emmelin, Muren, and Strömblad, 1954). This type of denervation is a preganglionic one. For the present investigation we have used the parotid gland, which can be both pre- and postganglionically denervated.

METHODS

The experiments were carried out on cats under chloralose anaesthesia (80 mg./kg. intravenously after preliminary ether). The parotid ducts were cannulated on both sides using cannulae which delivered 35 drops of distilled water/ml. Drops of saliva falling from the tip of the cannula were recorded on a smoked drum by a signal. Anticholinesterases were injected through the duct towards the gland. This has the advantage of an effect restricted to the injected gland, no interfering general effects are obtained, and the contralateral gland can be used as a control (Emmelin *et al.*, 1954). The following anticholinesterases were used: eserine, ethyl pyrophosphate (TEPP) and paraoxon (Mintacol, *p*-nitrophenyl-diethyl phosphate). These drugs, dissolved in 0.1 ml. saline solution, were injected through a fine rubber tube connected to the salivary cannula. Before the injection the tube was obstructed by a clip; the fluid was injected during 5 sec. and the clip removed 5 sec. later. Control injections of saline solution were made in the same way. Acetylcholine and methacholine were injected through a cannula in a femoral vein.

The parotid gland was parasympathetically decentralized (by destruction of Jacobson's anastomosis) or denervated (by section of the auriculo-temporalis nerves) as described by Strömblad (1955). Further technical details are given below.

RESULTS

Anticholinesterases were found to cause a secretion from the normally innervated parotid gland. Doses of 10 to 50 μg. of the drugs used were required to cause a secretion. One or two drops fell soon after the removal of the clip; this also occurred with control injections of saline solution. After 2 or 3 min., however, a flow started which continued for 10 to 15 min. after a dose of 10 μg. With 50 μg., the flow lasted for $\frac{1}{2}$ to 2 hr. The secretory effects of all three drugs tested were transient and doses repeated after some hours caused similar responses. Doses of the anticholinesterases slightly below those needed for secretion increased the secretory effects of acetylcholine and methacholine given intravenously. When a secretion caused by anticholinesterases had worn off the gland still showed an increased sensitivity to acetylcholine and methacholine for some time (Figs. 1 and 2), but this effect, too, was transient.

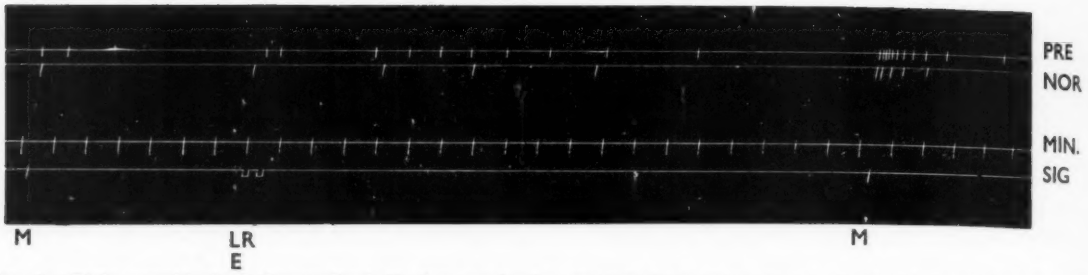

FIG. 1.—Right parotid gland decentralized 18 days previously. Records from above down: secretion from right gland (PRE) and from left gland (NOR); time in min.; signal (SIG); M=methacholine (2 μg./kg.) given intravenously; E=eserine (10 μg.) into the left (L) and into the right (R) duct.

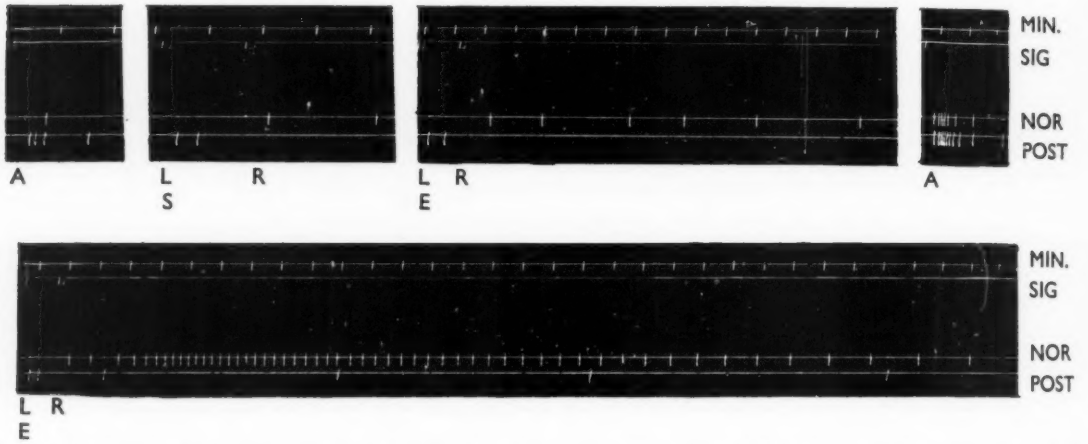

FIG. 2.—Left parotid gland denervated 18 days previously. From above down: time in min.; signal (SIG); secretion from right gland (NOR) and from left gland (POST); A=acetylcholine (2 μg./kg.) intravenously; S=0.1 ml. saline solution into the left (L) and into the right (R) duct; E=eserine (10 μg. in upper and 50 μg. in lower tracing) injected into the ducts.

After previous preganglionic, parasympathetic denervation the parotid gland still responded to anticholinesterases, as shown in the experiment shown in Fig. 1. In this cat the preganglionic fibres had been cut on the right side 18 days before the experiment. Eserine, 10 μg. injected through the ducts, caused a flow of saliva from both glands. The response of the decentralized gland was greater than that of the normally innervated one. As shown in the figure, this gland was supersensitive to methacholine. These observations on the parotid gland are in accord with those made previously on the submaxillary gland (Emmelin *et al.*, 1954).

Fig. 2 shows an experiment in which postganglionic, parasympathetic denervation of the parotid gland had been carried out by cutting the auriculo-temporal fibres on the left side 18 days previously. An injection of 10 μg. of eserine into the parotid duct caused a secretion from the normally innervated gland only; the effect on the left gland equalled that caused by saline solution. An injection of 50 μg. of eserine induced a flow of saliva from both glands, but the response of the denervated gland was much smaller than that of the normally innervated gland. This was the case in spite of the fact that the operation had caused a supersensitivity to acetylcholine, as shown in the upper part of Fig. 2.

When pre- and post-ganglionically denervated parotid glands were compared, the latter was found to be more sensitive to drugs such as acetylcholine and methacholine than the former. Nevertheless, anticholinesterases caused a bigger secretory effect on the decentralized than on the denervated gland.

In most of the cats the postganglionic fibres were cut 2 to 4 weeks before the acute experiment; the secretory effects of anticholinesterases on the denervated glands were in these cases invariably much smaller than on the contralateral, normally innervated glands. The same observation was made on a cat, operated on five days previously. Immediately after the operation, on the other hand, the denervated gland responded like a normal gland.

Special attention was paid to the small secretion caused by the injection of anticholinesterases into a gland, the auriculo-temporal nerves of which had been cut previously. Like the secretion caused from a normal gland, it was abolished by small doses of atropine, given intravenously. Similarly, it was abolished by a dose of cocaine (5 to 10 mg.), injected into the salivary duct, which did not affect the response of the secretory cells to acetylcholine or methacholine. In one experiment the facial nerve was cut, in another the superior cervical ganglion extirpated simultaneously with trans-section of the auriculo-temporal nerves. In both cases the usual small secretory response was obtained when anticholinesterases were given in the acute experiment some weeks later.

Discussion

When cats were treated for two or three weeks by daily subcutaneous injections of the anti-acetylcholine drug Hoechst 9980 (diphenyl-piperidino-ethyl-acetamide), a supersensitivity to chemical agents was found to develop in the submaxillary gland which exceeded and could be superimposed upon that obtained after preganglionic, parasympathetic denervation (Emmelin and Strömblad, 1957). Since this drug was found to be a remarkably specific acetylcholine antagonist, it was postulated that the treatment had caused a superimposed sensitization by excluding the action of acetylcholine released from the postganglionic endings. It was thus presumed that there is a release of acetylcholine from the postganglionic parasympathetic endings even when the postganglionic neurone has been disconnected from the central nervous system.

The present experiments were carried out in an attempt to find further evidence for such a release of acetylcholine.

Anticholinesterases, acting locally in the parotid gland, were found to cause a flow of saliva. This effect was obtained in a normally innervated gland and in accordance with previous experiments was not changed by acute preganglionic denervation (Emmelin *et al.*, 1954). After chronic preganglionic denervation a response was still obtained, and it was even bigger than that of a normally innervated gland. It seems reasonable to connect this with the fact that the effector cells were supersensitive to secretory drugs. The secretory effect of anticholinesterases is obviously not dependent on impulses from the central nervous system.

After chronic postganglionic, parasympathetic denervation, on the other hand, the secretory effect of anticholinesterases was very small in spite of the fact that the gland cells were supersensitive to drugs like acetylcholine and methacholine.

The secretory effects of the anticholinesterases were abolished by atropine. It cannot be excluded that the drugs exerted their effect by a direct, acetylcholine-like action, or by releasing acetylcholine. It is, however, generally assumed that anticholinesterases act mainly by causing an accumulation of acetylcholine, released in the course of physiological events. If so, the results of the present experiments would suggest that there is a liberation of acetylcholine from the postganglionic endings which is not due to impulses from the central nervous system. The finding that acute section of the auriculo-temporal fibres does not affect the secretory response to anticholinesterases would then indicate that such a release is not initiated from the cell body of the postganglionic neurone, but rather is due to a process analogous to that assumed to operate at the endings of somatomotor fibres.

Five days or more after section of the auriculo-temporal nerves, when the fibres could be assumed to have degenerated, anticholinesterases had a small secretory effect only. This seems compatible with the idea that the drugs acted mainly by an acetylcholine mechanism; if they had some direct action exclusively one would rather expect them to have an enhanced effect on the supersensitive gland cells.

The small secretory effect of anticholinesterases remaining after previous postganglionic denervation could be explained in different ways. It could be a slight direct effect on the supersensitive gland cells or it could be due to preserved acetylcholine. Such acetylcholine might be of non-nervous origin, for instance like that found in the gut by Feldberg and Lin (1949). These authors observed, however, a release of acetylcholine even after administration of cocaine, whereas in our experiments cocaine abolished the effect of anticholinesterases. Possibly some cholinergic fibres to the gland were still intact after the operation on the postganglionic nerves, but attempts to eliminate such fibres by section of the facial nerve or excision of the superior cervical ganglion were unsuccessful. It may be added that Chang and Gaddum (1933) showed that acetylcholine is present in the parotid gland after previous section of the auriculo-temporal nerves although in reduced quantity.

We are grateful to Dr. B. Holmstedt, Stockholm, for a gift of ethyl pyrophosphate and Mintacol.

Technical assistance was given by Miss Ursula Delfs.

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A METHOD FOR RECORDING THE EFFECTS OF ANTHELMINTICS ON THE MOVEMENTS OF *ASCARIS LUMBRICOIDES*

BY

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Adult roundworms (*Ascaris lumbricoides*) obtained from pigs were suspended in modified Tyrode solution at 37° in fine nylon stockings, and the movements of the stockings were recorded. This method of suspension avoided damage to the cuticle or occlusion of the alimentary tract of the worm.

Piperazine salts caused gradual narcosis, the time of onset of which was related linearly to the logarithm of the concentration of drug in the bath. The method is suitable for the assay of preparations of piperazine. Tetrachlorethylene, hexylresorcinol, and oil of chenopodium all caused stimulation of movement before the worm was immobilized. Santonin caused incoordination of the movements of the anterior end of the worm; the movements of the body were apparently unaffected. Piperazine is the safest preparation available for the treatment of ascariasis.

Baldwin (1943) studied the effect of anthelmintics on the movements of isolated portions of *Ascaris lumbricoides* prepared from the anterior and intermediate parts of the worm and suspended in nutrient fluid in an isolated organ bath. Baldwin and Moyle (1949) investigated the action of drugs on portions of worm from which the cuticle had been removed and the muscle exposed. More recently, Norton and de Beer (1957) have used similar muscle preparations for the investigation of the action of piperazine. Preparations of this kind do not survive long *in vitro* and they give little indication of what may happen to a worm when the anthelmintic reaches it through the alimentary canal of the host in which it lives. Some drugs, such as piperazine, act slowly (Standen, 1955; Brown, Chan and Hussey, 1956) and are probably ingested by the worm. Baldwin (1943) recognized these difficulties.

The main problem of recording the movements of whole worms is that of harnessing them to the recording lever in such a way that the cuticle is not damaged by stitches, the alimentary canal is not constricted by ligatures and the movements of the worm are unimpeded. A suitable method has been devised in which the worm is introduced into a fine nylon stocking and the movements of the stocking are recorded.

METHODS

Ascaris lumbricoides. — Worms were obtained from pigs at the slaughterhouse and transported to the laboratory in a vacuum flask containing modified Tyrode solution at 37° (NaCl 0.8%, KCl 0.02%, $CaCl_2$ 0.02%, $MgCl_2$ 0.01%, $NaHCO_3$ 0.015%, Na_2HPO_4 0.05%, glucose 0.5%). They were stored in an incubator in covered glass pans of the solution, which was changed daily.

Stockings.—Tubes about 16 in. long and $\frac{3}{16}$ to $\frac{1}{4}$ in. in diameter were prepared from discarded nylon stockings (12 denier, 60 gauge); the seam was made in fine cotton with a sewing machine. One end of the stocking was left open, the other was tapered to the shape of the anterior end of the worm and was closed. Stockings of this size are suitable for adult female worms; male worms, which are smaller, require narrower and shorter stockings.

The worm was introduced into the stocking in a dish of warm Tyrode solution. The open end was closed behind the worm with a rubber band; the band was attached to a sinker made from a sealed glass ampoule (5 ml.) filled with lead shot. The closed (anterior) end of the stocking was attached to a length of fine cotton which was used to lower the worm into a bath 16 in. long and $2\frac{1}{2}$ in. in diameter (capacity about 400 ml.) containing Tyrode solution maintained at 37°. A small cap made from black glass tubing was threaded over the cotton to shade the light-sensitive anterior tip of the worm. The cotton was

attached to a frontal writing lever with sufficient counterweight to keep the worm upright in the bath. A diagram of the apparatus is shown in Fig. 1. Movements were recorded on a slow drum without magnification. No oxygen was bubbled through the nutrient solution ; *Ascaris* is an anaerobe.

In some experiments a bath was used which restricted the movements of all but the anterior ½ in. of the worm. This was made from a length of ¼ in. bore glass tubing fused to a wide tube above. The body of the worm in its stocking was imprisoned in the narrow tube ; the anterior tip was free to move and the movements were recorded with a light lever magnifying ten times.

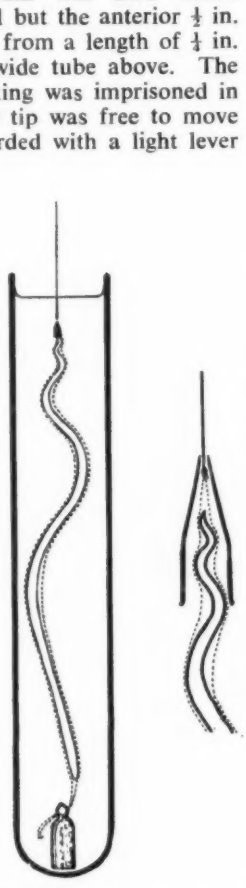

FIG. 1.—Worm in stocking suspended in a bath of Tyrode solution. The lower end of the stocking is anchored by an ampoule full of lead shot and the upper end is shaded by a small cap of black glass.

Drugs.—Water-soluble anthelmintics were dissolved in nutrient medium and added to the bath. Those which were insoluble were emulsified or brought into solution with medium containing 0.5% sodium tauroglycocholate.

At least ½ hr. was allowed for a worm to settle down before adding a drug. A continuous record was taken for 1 hr. after adding the drug ; with slow-acting substances, tracings were recorded for ¼ hr. periods once every hour for the following 24 hr. This was arranged by interposing a Sangamo-Weston interval-timer in the mains supply to the kymograph motor. Drugs were left in contact with the worms for the whole period of observation.

RESULTS

Normal Movements.—The worm moved by arching the body in the dorsiventral plane, first in one direction and then in the other. Movements of the adult female were most vigorous in the anterior ¼ to ½ of the body ; the posterior part, occupied by the gravid uterus, was relatively quiescent unless stimulated by drugs. The arching of the body took place fairly regularly at intervals of 2 to 3 min. A sinuous wave appeared to run forwards along the anterior part of the worm, decreasing in both amplitude and wavelength as it approached the anterior end. The extreme tip of the worm was very active and carried out independent searching movements. The normal tracing (Fig. 2) shows the arching movements as large excursions, with the more rapid movements of the tip superimposed. The function of the searching movements of the anterior end was sometimes demonstrated in a stocking with a small hole in it. If the hole was large enough to admit the extreme tip of the worm, vigorous sinuous movements of the body thrust it through ; the constriction formed around the worm by the edges of the hole stimulated it to even greater activity and it soon enlarged the hole sufficiently for the whole body to pass through. Observation of these powerful movements makes clear the method by which the worm can maintain its position in face of the peristaltic waves in the small intestine of the host, and the reason why displaced worms are occasionally found in the appendix, the bile duct and the nose. In early experiments, before a black glass cap was used to cover the anterior end of the worm, the creature sometimes doubled back in the stocking, often making two or three folds of its body in a part of the stocking which would stretch enough to hold them ; sometimes it reversed its position completely. These manœuvres were always accompanied by very vigorous contractions. If the anterior end was shaded the worm always remained in its original position.

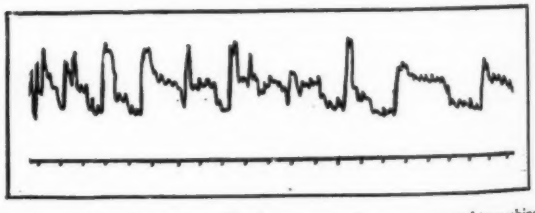

FIG. 2.—Normal tracing. The large excursions correspond to arching movements of the body, the small excursions to searching movements of the anterior end. Time, min.

The use of a sinker to anchor the stocking to the bottom of the tube was a more satisfactory method than attachment to the lower end of a

hooked glass rod. Worms sometimes wound themselves around the rod, used it as a support and became relatively quiescent.

Piperazine.—The effect of a concentration of 1:250 of piperazine citrate in the bath is shown in Fig. 3. The worm was slowly narcotized and after about 6 hr. its movements ceased. Lower concentrations of drug also produced narcosis but took longer to do so. Fig. 4 shows that the mean time taken to immobilize the worms was linearly related to the logarithm of the concentration of drug in the bath. The activity of piperazine citrate was equal to that of other salts, such as the adipate and phosphate, in amounts containing an equivalent quantity of base. Piperazine adipate is completely soluble at 1:250. Pharmaceutical preparations of piperazine in syrup also had activity proportional to their content of piperazine base; syrup alone had no significant effect. No significant stimulation of movement by piperazine was observed in experiments with several hundred worms. Worms narcotized with piperazine began to move again when immersed in fresh medium but never recovered their original vigour. Male worms were 10 times as sensitive to piperazine as female worms.

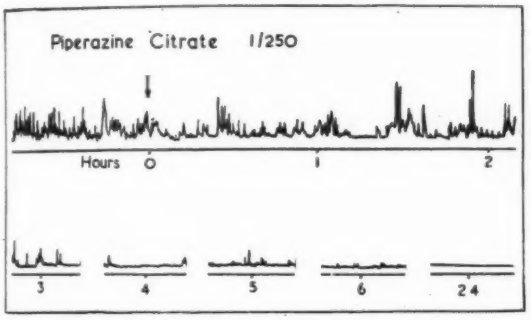

FIG. 3.—Effect of piperazine citrate 1:250. The movements gradually diminished and the worm became quiescent in about 6 hr. There was no stimulation.

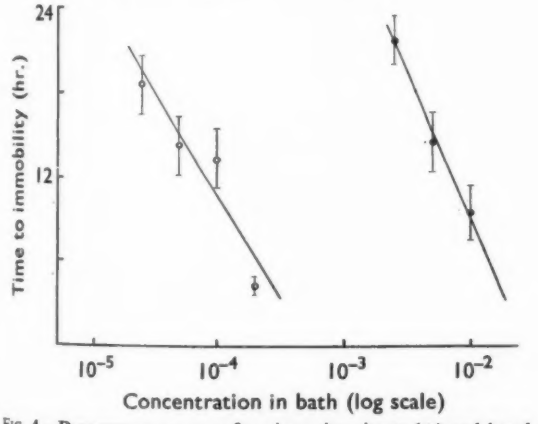

FIG. 4.—Dose-response curves for piperazine citrate (●) and hexylresorcinol (O). Groups of 10 worms were used at each concentration of drugs and the mean time to immobility (max. 24 hr.) was plotted against log concentration. The vertical lines indicate the standard errors of the means. Calculation of relative potency by the method of Specification 911 of the British Standards Institute (1940) shows hexylresorcinol to be 98.8 times as active as piperazine citrate. Tests for linearity and parallelism were satisfactory and the true fiducial limits of the activity ratio were 66.0 to 158.5% ($P=0.95$).

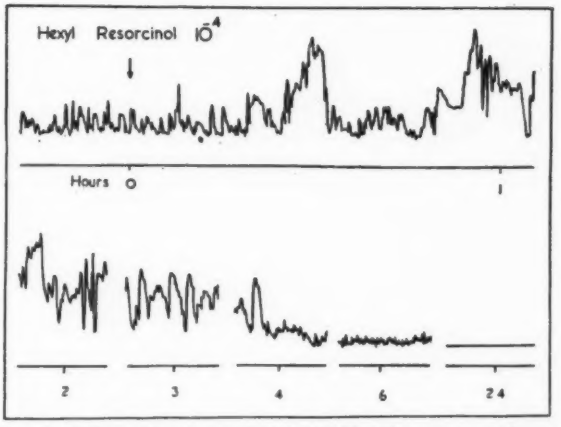

FIG. 5.—Effect of hexylresorcinol 1: 10,000. The worm was immobilized, but only after several hours of increased activity.

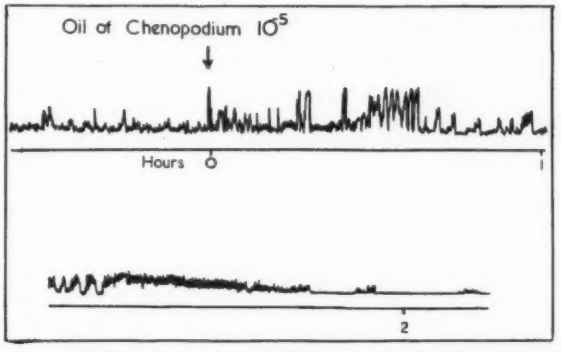

FIG. 6.—Effect of oil of chenopodium 1: 100,000. The worm was immobilized in 2 hr. after a period of slightly greater activity than normal.

Hexylresorcinol.—This drug was about 100 times as active as piperazine (Fig. 4) but caused marked stimulation before the worm became immobile (Fig. 5). Worms which had been immobilized with hexylresorcinol did not recover in fresh medium. Thymol had the same type of action as hexylresorcinol but was much less potent.

Oil of Chenopodium.—A concentration of 10^{-4} of oil of chenopodium, emulsified with bile salts, brought the movements of the worm to a standstill in a few minutes. Lower concentrations took longer and sometimes caused preliminary stimulation (Fig. 6).

Santonin.—Santonin had no detectable effect on the arching movements of *Ascaris*; 24-hr. tracings made by worms in a concentration of 10^{-3} of santonin solubilized with bile salts were similar to those made by normal worms. On the other hand, the searching activities of the anterior end of the worm were impaired very soon after the drug was added to the bath. The movements became feeble and incoordinated. When the arching movements were suppressed by enclosing the worm in a narrow tube, only the movements of the anterior end were recorded on the drum; on the addition of santonin these movements became too feeble to move the light lever (Fig. 7).

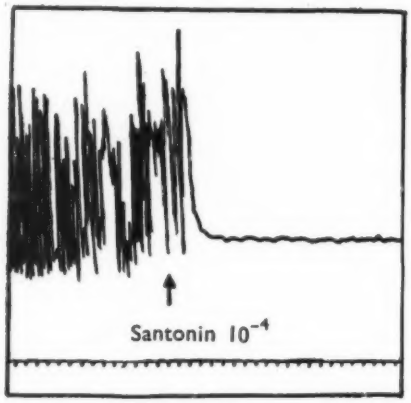

FIG. 7.—Effect of santonin 1: 10,000 on the movements of the anterior ½ in. of the worm. The movements of the body of the worm were suppressed by enclosure in a narrow tube; only the tip was free to move. Time, min.

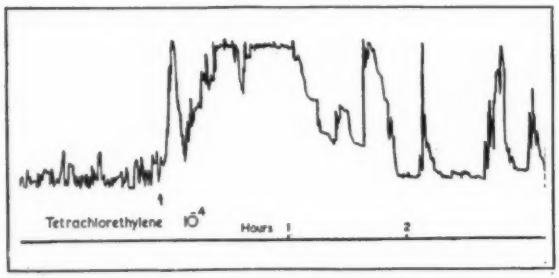

FIG. 8.—Effect of tetrachlorethylene 1: 10,000. The worm was stimulated to violent and incoordinated activity.

Tetrachlorethylene.—An emulsion of tetrachlorethylene stimulated the worm to violent, convulsive activity (Fig. 8). The posterior part of the female worm, usually quiescent, also convulsed and often remained for many minutes in a state of contracture in bizarre looped or angled attitudes. The worms did not recover in fresh medium.

DISCUSSION

These observations show that although piperazine is less potent, weight for weight, than the other anthelmintics tested, it has more desirable properties than the other drugs at present available for the treatment of ascariasis. It takes several hours to exert its action, and produces gradual narcotization without any preliminary phase of stimulation; it has a wide margin of safety in man. The effectiveness of a single large dose of piperazine as an ascarifuge has been shown by Goodwin and Standen (1954, 1958).

Small children in the tropics frequently carry a burden of 30 or 40 adult roundworms, and some have several hundreds. An adult female is 12 to 14 in. in length and ¼ in. in diameter; a group of 50 females occupies a volume of about 200 ml. The worms are strong and muscular, and it is not surprising that drugs such as tetrachlorethylene and hexylresorcinol which stimulate them to violent activity sometimes cause obstruction or perforation of the intestine when given to patients with heavy worm burdens. Sappenfield, Swartzwelder, and Miller (1957) have reported the successful use of piperazine citrate given by mouth or by intestinal tube to children less than 4½ years of age suffering from partial intestinal obstruction caused by roundworms. Surgical intervention was unnecessary. Pena-Chavarria, Lizano, and Xirinachs (1957) have used the drug to obviate the dangers of perforation by roundworms in patients with typhoid fever.

Norton and de Beer (1957) showed that 1:20,000 of piperazine partially inhibits the contraction produced by acetylcholine in preparations of *Ascaris* in which the muscle is exposed to the action of the drug. Electrically-stimulated contractions of the muscle were unaffected by piperazine; the drug appeared to have an action on neuromuscular transmission in the worm resembling that of tubocurarine in the mammal.

The higher sensitivity of male worms to the action of piperazine explains some observations made in the course of field trials in the Gambia (Goodwin and Standen, 1958). A single dose containing the equivalent of 4 g. of piperazine hexahydrate removed all the ascarides from more than 80% of patients. The remaining patients were freed from most of their worms, but a few eggs were still present in the faeces a week after treatment. Most of these eggs were infertile, showing that the worms that remained were mostly unmated females.

Oil of chenopodium, although a very potent ascaricide, is toxic to the liver of the host and is

dangerous to use in patients with obstruction caused by roundworms.

The action of santonin was studied by Baldwin (1943) on fragments of *Ascaris*. He observed that the drug affected preparations of the anterior end, but had no action on preparations from the intermediate part of the worm. He concluded that santonin exerted its effect on the cephalic ganglia. These observations have been confirmed on whole undamaged worms, and it is clear that the action of santonin as a vermifuge must depend upon this incoordinating action. It is likely that, unless the searching activity of the anterior end can find and penetrate the central canal of an advancing peristaltic wave, the arching movements of the body which brace it against the walls of the gut are not sufficiently well-directed for it to maintain its position. A worm which has been affected by santonin therefore, like one narcotized by piperazine, becomes swept along the gut by the peristaltic contractions and is expelled.

The skilled assistance of Mr. W. H. G. Richards is gratefully acknowledged.

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THE TOXICITY OF 2-HYDROXYIMINOMETHYL-*N*-METHYLPYRIDINIUM METHANESULPHONATE (P2S)

BY

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The toxicity of 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (P2S) has been determined in a number of species by various routes. It is approximately equally toxic in the rat, mouse, and guinea-pig. It is much more toxic in the dog. Atropine influences the toxicity of P2S differently in different species. From the results obtained attempts have been made to assess the maximum safe dose which can be given intramuscularly to man.

The value of oximes administered with atropine in the treatment of organophosphate poisoning has been demonstrated by Kewitz and Wilson (1956), Kewitz, Wilson and Nachmansohn (1956), Hobbiger (1957), Wills, Kunkel, Brown and Groblewski (1957), Askew, Davies, and Green (1957), and Askew (1957). The toxicity of the more effective oximes is therefore a matter of some importance, particularly if they are to be used in man.

No systematic studies upon the toxicity of oximes have been described, although Askew (personal communication) screened some of them in rats with a maximum dose of 150 mg./kg. i.p. as a preliminary to examining their efficacy in organophosphate poisoning. Most of the oximes she examined were non-toxic at 150 mg./kg., but di*iso*nitrosoacetone and *p*-methoxy*iso*nitrosoacetone were lethal at 30 mg./kg., and glyoxime, *iso*nitrosoacetylacetone, mono*iso*nitrosoacetone and phenylglyoxime at 100 mg./kg. Askew also found that signs of oxime poisoning were similar for all the compounds studied and consisted of marked lethargy, followed by prostration, muscular tremors and loss of reflexes. Signs of poisoning usually developed within 15 min. of injection. When animals survived, recovery began within an hour. Glyoxime was exceptional in that poisoning was delayed for several hours.

Dultz, Epstein, Freeman, Grey, and Weil (1957) determined the LD50 values for some oximes given intraperitoneally to mice; they varied from 20 mg./kg. with di*iso*nitrosoacetone to about 4,000 mg./kg. with acetone oxime. Di*iso*nitrosoacetone, *iso*nitrosoacetylacetone, *iso*nitrosoacetophenone, *iso*nitrosoacetanilide and 1-diethylamino-2:3-butanedione 2-oxime methiodide were all toxic at less than 150 mg./kg.

Three oximes have proved particularly promising as antidotes to organophosphate poisoning. These were mono*iso*nitrosoacetone (MINA), diacetyl monoxime (DAM) and pyridine-2-aldoxime methiodide (2-hydroxyiminomethyl-*N*-methylpyridinium iodide; P2AM), and their toxicities have been studied more fully, but still no systematic studies of the effect of species and route of administration upon their toxicity have been reported. MINA has been examined in greatest detail. The intraperitoneal LD50 value for mice is 150 mg./kg. (Dultz *et al.*, 1957), but the corresponding values for male and female rats are 50 mg./kg. and 75 mg./kg. respectively (unpublished observations). MINA is toxic because it is converted in the body into cyanide, a reaction which is probably specific to 2-oxo-aldoximes (Askew, Davies, Green, and Holmes, 1956).

DAM is relatively non-toxic, and its toxicity by the intraperitoneal route to mice is 900 mg./kg. (Dultz *et al.*, 1957). No other results are available. Although P2AM is the most promising of the three compounds therapeutically, information concerning its toxicity is very limited. Thus Kewitz and Wilson (1956) give the LD50 for mice as 136 ± 6 mg./kg. (i.p.) and add that with doses of this order death occurs in about 20 min. Askew (unpublished observation) showed that 100 mg./kg. (i.p.) of P2AM was non-lethal to rats and indeed did not seem to cause any obvious effects.

From the above it is clear that toxicity estimates on oximes are scanty and incoordinated.

P2AM is relatively insoluble, so that its use in large mammals might well be limited. The sub-

stitution of a methanesulphonate for the iodide of P2AM gives a much more soluble substance, 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (P2S) without loss of therapeutic efficacy.

Materials and Methods

Three oximes were used in this investigation. Pyridine-2-aldoxime (P2A), 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (pyridine-2-aldoxime methiodide; P2AM) and 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (P2S).

Toxicity was determined in female rats (180 to 200 g.), mice (18 to 22 g.), rabbits (2 to 3 kg.), and guinea-pigs (350 to 400 g.). Both sexes were used in determinations on monkeys (3 to 4 kg.).

All solutions were made up in water and the *p*H adjusted to 7.4 by the addition of NaOH. In any one determination of the LD50 the volume of the injection/kg. body weight was constant. Owing to differences in the weight of different species this volume varied from species to species and also with the route of administration. The volume used is recorded in the appropriate section of the text.

LD50 values were calculated by the method of moving averages (Thompson, 1947) using the Tables for convenient calculation of the median effective dose (Weil, 1952), except where indicated in the text. A four-point assay was used and six or four animals were used at each point.

Results

The Signs of Poisoning by P2S.—Sub-lethal intramuscular injections of P2S produce lethargy and increased respiration. Lethal doses cause muscular tremors, convulsions, dyspnoea, cyanosis, and death. Doses of 2 to 3 × LD50 produce death in 3 to 4 min. when given intravenously, and in 20 to 30 min. intramuscularly.

The minimum doses at which onset of signs of poisoning were seen in various species are given in Table I together with the maximum doses at which no signs were observed.

Table I

THE APPEARANCE OF SIGNS IN VARIOUS SPECIES FOLLOWING INTRAMUSCULAR INJECTION OF P2S

Species	Maximum Dose at which NO Signs were Observed (mg./kg.) (1)	Minimum Dose at which Signs were Noted (mg./kg.) (2)	Severity of Signs at Minimum Dose (see Col. 2) (3)
Mouse ..	100	120	Very slight
Rat ..	126	159	,, ,,
Guinea-pig	159	200	,, ,,
Rabbit ..	126	159	,, ,,
Dog ..	30	60	Marked
Monkey ..	—	200	,,
Sheep ..	60	c. 80	Slight

In the mouse, rat, guinea-pig and rabbit, doses of 100 mg./kg. were given without any obvious toxic effects, although slight signs, such as lethargy and increased rate of respiration, were obvious when the dose was increased by about a quarter over that shown in column 1.

The minimum dose given to monkeys was 200 mg./kg. Although there were no deaths, it produced restlessness, lethargy and tremors in each animal. Two of the four animals collapsed for from 10 to 20 min.

The first indications of toxic effects in sheep were seen with approximately 80 mg./kg., but these effects were slight.

The dog was the most sensitive mammal studied. No signs of poisoning occurred at 30 mg./kg., but at 60 mg./kg. they were marked, consisting of gasping respiration, tremors, vomiting, inability to hold up its head, sometimes followed by collapse. Recovery was complete in 1 hr.

In rats killed with P2S, moderate to severe cyanosis was seen in the lungs and abdominal organs. Histologically the lungs showed generalized congestion with areas of collapse and some haemorrhages; the kidneys and the liver were also congested.

Rats given six intramuscular injections of 80 mg./kg. at approximately 1 hr. intervals showed no obvious ill effects other than slight lethargy. Microscopic examination of the tissues on the next day showed slight congestion of the lungs, liver, spleen and kidney, in one animal. There was, however, some damage to muscles at the site of injection, but there was no visible damage to the muscles in animals killed a fortnight later.

The Toxicity of P2S in Various Species by Various Routes. — The intravenous and intramuscular LD50 values of P2S in the mouse, rat, rabbit and guinea-pig, together with the LD50 value by the intramuscular route in the monkey, are recorded in Table II.

Table II

INTRAVENOUS AND INTRAMUSCULAR TOXICITIES OF P2S TO VARIOUS SPECIES

24 animals were used in each determination of LD50 except in the case of monkeys, where only 16 animals were employed.

Species	Intravenous			Intramuscular		
	Vol. of Soln. Injected ml./kg.	mg./kg. LD50	mg./kg. Limits (95%)	Vol. of Soln. Injected ml./kg.	mg./kg. LD50	mg./kg. Limits (95%)
Mice ..	10·0	122	111–135	1·0	231	215–248
Rats ..	10·0	109	96–123	1·0	218	208–227
Rabbits ..	1·0	147	126–169	1·0	233	203–269
Guinea-pigs				1·0	305	268–342
Monkeys ..				1·0	356	303–419

P2S, intravenously, is equally toxic in the mouse, rat and the rabbit, the LD50 values lying between 110 and 150 mg./kg.

The intramuscular LD50 values for mice, rats and rabbits are about twice the intravenous value (approximately 230 mg./kg.) but are less than the intramuscular LD50 for monkeys which is 356 mg./kg.

These differences in the intramuscular and intravenous LD50 values prompted a more detailed examination of the effect of route of administration upon the toxicity of P2S. Table III gives the LD50 estimates obtained by administering the oxime to rats and mice in various ways.

TABLE III

VARIATION OF TOXICITY OF P2S IN RATS AND MICE WITH ROUTE OF ADMINISTRATION

24 animals were used in each determination of LD50.

Route of Admin.	Rats			Mice		
	Vol. of Injection (ml./kg.)	mg./kg.		Vol. of Injection (ml./kg.)	mg./kg.	
		LD50	Limits		LD50	Limits
i.v. ..	10·0	109	96–123	10·0	122	111–135
i.m. ..	1·0	218	208–227	1·0	231	215–248
s.c. Flank	4·0	527	438–635	10·0	191	159–230
,, Scruff	4·0	332	282–392	10·0	165	145–188
i.p. ..	10·0	262	237–288	10·0	216	188–249
Oral ..	2·5	>800		5·0	>2000	

Excluding oral administration, LD50 values varied in the rat from 109 mg./kg. intravenously to 527 mg./kg. when the oxime was given subcutaneously into the flank. When the oxime was injected subcutaneously into the scruff of the neck the toxicity was significantly less (332 mg./kg.).

In mice such variations were less marked. In fact, differences in the LD50 values by the parenteral routes were not significant.

TABLE IV

THE RELATIVE TOXICITY OF P2S BY A GIVEN ROUTE, EXPRESSED IN TERMS OF THE INTRAVENOUS TOXICITY

Route of Administration	Relative Toxicity	
	Rats	Mice
i.v.	1=109	1=122
i.m./i.v.	2·0	1·9
s.c. Flank/i.v.	4·9	1·6
,, Scruff/i.v.	3·0	1·4
i.p./i.v.	2·4	1·5

The effect of route of administration in these two species is best seen by a comparison of the toxicity by a given route, expressed in terms of the intravenous toxicity (see Table IV). In rats this varies from 2.0 to 4.9, whereas in mice it is between 1.4 and 1.9.

The Influence of Sex upon the Toxicity of P2S.—In view of the sex difference found in the toxicity of MINA to rats, intraperitoneal LD50 values were determined in both sexes in rats and mice. These are given in Table V.

No significant difference was demonstrated by this route.

TABLE V

INTRAPERITONEAL TOXICITY OF P2S TO MALE AND FEMALE RATS AND MICE

The volume of P2S injection was 10·0 ml./kg. in both species. 24 animals were used in each LD50 determination.

	Male		Female	
	mg./kg.		mg./kg.	
	LD50	Limits	LD50	Limits
Rats ..	317	260–387	262	237–288
Mice ..	147	130–167	178	146–217

A Comparison of the Toxicity of Some Derivatives of Pyridine-2-Aldoxime (P2A).—In the course of the general programme, the toxicities of three closely related compounds, P2A, P2AM and P2S, have been compared. Intraperitoneal LD50 values are given in Table VI for rats and mice. All three compounds were equally lethal to rats, but both P2AM and P2S were significantly more toxic than P2A to mice. Again P2AM was less toxic to rats than to mice.

TABLE VI

A COMPARISON OF THE INTRAPERITONEAL TOXICITY OF DERIVATIVES OF PYRIDINE-2-ALDOXIME (P2A)

The volume of P2S used was 10·0 ml./kg. and of P2A and P2AM was 20·0 ml./kg. 24 animals were used in each LD50 determination.

Compound	Rats		Mice	
	mg./kg.		mg./kg.	
	LD50	Limits	LD50	Limits.
P2A ..	299	237–377	305	268–347
P2AM ..	305	277–336	233	216–252
P2S ..	262	237–288	216	188–249

Although the LD50 values expressed in mg./kg. for each of these compounds does not differ markedly, a major difference in toxicity becomes apparent when the mg./kg. values are converted to moles/kg. The molecular weight of P2A is only half that of P2AM or P2S. Expressed in this way, P2A is only half the toxicity of the others. This difference in toxicity is also shown by a comparison of their speed of action (Table VII).

In mice a "sure lethal" dose of approximately $2 \times$ LD50 given intravenously of either P2AM or P2S resulted in immediate convulsions with death from 1 to 6 min. With P2A, the shortest time to

death observed in a series of 35 animals was 120 min. and the longest 280 (Table VII).

TABLE VII

THE TIME TO DEATH IN MICE POISONED WITH ABOUT 2 × LD50 OF P2A, P2AM, AND P2S

Compound	No. of Animals	Mean Time to Death (min.)	Range (min.)	Development of Symptoms
P2A	35	200	120–280	Immediate collapse: rapid recovery in 1–10 min. Death in about 3 hr.
P2AM	15	2	1–6	Immediate convulsion with rapid onset of death
P2S	10	1	1–3	Immediate convulsion with rapid onset of death

The Effect of Atropine upon the Toxicity of P2S.—As an antidote in alkyl phosphate (anticholinesterase) poisoning, oximes are only markedly effective in conjunction with atropine. The toxicity of P2S has therefore been determined in mice, rats and guinea-pigs in the presence of atropine. A standard dose of 17.4 mg./kg. atropine sulphate was given intramuscularly, together with various doses of P2S, and regression lines constructed. The constants of such curves are shown in Table VIII.

TABLE VIII

CONSTANTS OF PROBIT-KILLED/LOG_{10} DOSE REGRESSION LINES FOR P2S IN DIFFERENT SPECIES AND IN THE PRESENCE AND ABSENCE OF ATROPINE

P2S plus atropine sulphate (17·4 mg./kg.) given together i.m. 80 animals were used for the construction of each probit line.

Species	Agent	Regression Coefficient ± Standard Error	LD50	95% Fiducial Limits LD50	Probability Level of χ^2 for Parallelism
Rat ..	P2S	14·8 ± 2·26	218	208–227	70%
	P2S + Atrop.		319	304–338	
Guinea-pig	P2S	11·0 ± 2·10	305	268–342	70%
	P2S + Atrop.		238	208–260	
Mouse	P2S	12·7 ± 1·31	231	215–248	40%
	P2S + Atrop.		218	204–233	

Three interesting points are apparent from Table VIII. First, the slopes of the regressions are steep. This is important, for, as will be shown later, it indicates that a relatively small increase below the LD50 results in doses whose lethal effects are considerably reduced. Secondly, regressions are essentially parallel for mice, rats and guinea-pigs. Atropine does not alter this parallelism. Thirdly, the effect of atropine upon the toxicity of P2S varies with the species. Thus, in rats, atropine raises the LD50 value by not more than about 20%, whilst in mice it has no effect.

DISCUSSION

The object of this work was to obtain information about the toxicity of P2S so that the likely maximum safe dose for man might be assessed.

Death has been used as the criterion of toxicity. Probit/log. dose regression lines have been constructed for rats, guinea-pigs and mice and, by assuming linearity, extrapolations to the calculated lines have been made. The results of these calculations are shown in Table IX where some theoretical dose/response relationships are given for the extreme end of the lines quoted in this Table.

TABLE IX

THEORETICAL DOSE/RESPONSE RELATIONSHIP OF I.M. INJECTED P2S AT THE EXTREME ENDS OF THE LINES. QUOTED IN TABLE VIII

Lethal Expectation	Rat (mg./kg.)		Guinea-pig (mg./kg.)		Mouse (mg./kg.)	
	LD	Limits	LD	Limits	LD	Limits
1/1 = LD50	218	208–227	305	268–342	231	215–248
1/100 ..	152	108–152	187	120–226	151	139–166
1/1,000 ..	135	94–151	160	91–201	132	118–147
1/10,000	122	83–132	140	71–183	118	103–134

In Table I the minimum observed dose is given at which signs were recorded and these values are by no means inconsistent with those in Table IX. In each species there is only a one in hundred chance of death at doses which cause the first signs of poisoning. For example, in rats given 150 mg./kg. the expectation of death is 1/100; very slight toxic effects were seen with 159 mg./kg. In the guinea-pig the 1/100 lethal probability occurs at 187 mg./kg. and again slight signs of poisoning were noted at 200 mg./kg. In mice the corresponding figures were 151 and 120 mg./kg. respectively. Although lethal probabilities are quoted at the 1/10,000 level, such estimates must be quite theoretical, since, at this level, death from other causes may well occur and cannot easily be distinguished from those attributable to P2S.

The fact that oximes will almost certainly be used with atropine also complicates the issue. However, since the probit/log. dose regression lines are essentially parallel not only for a given species in the presence and absence of atropine, but also for different species, provided that it is known in which direction atropine influences the toxicity in a given species, an appropriate adjustment in such estimates of "safe" dose can be made.

Although estimates of the intramuscular LD50 of P2S have been made for both the monkey and the dog, the number of animals upon which such estimates have been based are too few to per-

mit the construction of adequate dose/response curves. However, the fact that such lines are parallel for the rat, guinea-pig and mouse suggests that some approximate estimates can be made if it is permissible to assume that for monkeys and dogs the lines are also parallel.

Intramuscular LD50 values of P2S for the dog have only been assessed as a result of few observations and a provisional value of 70 to 80 mg./kg. has been given. On this basis, the dog is markedly more sensitive than any of the other species. The 1/100 lethal probability level is approximately two-thirds of the LD50 and, as has been shown, is the dose level at which symptoms first tend to appear. Two-thirds the LD50 value in the dog is about 50 mg./kg. At 60 mg./kg. poisoning was marked.

Approximately half the LD50 is the dose at which the chances of death are only 1/10,000 and at this dose no signs of poisoning would be expected. Half the LD50 is about 37 mg./kg. and at 30 mg./kg. there were no signs in any of the dogs so treated. These observations were made in the absence of atropine.

The LD50 for the monkey is greater than in any of the other species studied so that it does not seem illogical to assume that the safe dose in this species should not be less than either of the three in which more detailed studies have been carried out.

The value of estimates of the "safe" dose such as have been made above must lie in the use to which they are to be applied. Obviously they should not be treated as estimates of high precision, but they are a guide to dose levels above which P2S may only be given with the possibility of unpleasant side-effects and even death. However, in practice the choice of dose must depend upon whether it is to be given prophylactically or therapeutically. If the former it must be free from any lethal or incapacitating effects: in the latter, since the urgent requirement is saving the life of a seriously poisoned person, temporary inconvenience and even some risk of death from the oxime may be acceptable.

In general, then, it may be stated that doses of 100 mg./kg. intramuscularly of P2S may be given to the rat, mouse, guinea-pig or monkey with a lethal probability of less than 1/1,000. The dog appears to be much more sensitive, but the results are very much less reliable.

However, other factors tend to limit the size of a single dose. Thus therapeutically it is shown elsewhere that in the fully atropinized animal there is an optimum dose of P2S, approximately 30 mg./kg. intramuscularly beyond which there is no proportional increase in effectiveness. For convenient use in the field the total volume of injection must be limited. Again 30 mg./kg. appears to be indicated as a probable upper limit. Thus all the available results at the moment suggest that an intramuscular dose of 30 mg./kg. of P2S may be given to man without any serious side-effects and with a negligible risk of death.

These assessments have been arrived at without considering the effect of atropine upon the toxicity of P2S and this may, as shown, materially alter the picture.

We wish to express our great thanks to Mr. S. Peto of M.R.E. for help and advice on the statistical aspects of this paper. Our warm thanks are also due to Dr. A. L. Green for supplies of all the oximes used in this work. We also wish to acknowledge the technical help of Miss June Petts and Mr. R. C. Elliott.

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QUANTITATIVE ASSAYS OF OXYTOCIC DRUGS ON THE HUMAN POSTPARTUM UTERUS

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Quantitative assays of oxytocic drugs were performed on the postpartum human uterus by means of external tocography. Since, owing to the rapid involution of the uterus, only two doses could be given to each patient, the assays were designed as incomplete randomized blocks of 2. A dose/response curve for ergometrine was established, and a comparative assay of ergometrine and methyl ergometrine carried out, the design and analysis of which is described in detail.

Assays of oxytocic drugs on the animal uterus or on isolated strips of the human uterus frequently give a misleading indication of their potency in clinical use, and it is thus desirable to test these drugs on the intact human uterus. There are basically two methods of tocography that can be used to assay drugs on the human uterus, namely the internal and the external methods. The internal methods are in some ways more reliable since the pressures inside the uterus are directly measured, but they have the disadvantages that they are potentially dangerous and cannot easily be used on a sufficient scale to give results which are statistically valid. The external methods utilize an instrument measuring through the abdominal wall the hardening of the uterus during contraction. The advantage of the external method is that it does not in any way endanger the patient, and in fact causes no or practically no discomfort, nor does the method itself modify uterine activity.

Although external tocography has been used to study the effect of drugs during pregnancy and parturition this method has not apparently been used for the quantitative assay of oxytocic drugs after delivery. Because of the simplicity and safety of external recording and despite the apparent difficulties of obtaining satisfactory results from the involuting uterus in many difficult patients, we set out to use this method to establish the dose/response relationship for small doses of ergometrine, and subsequently used the method to compare quantitatively the effects of ergometrine and methyl ergometrine.

An attempt was made to design the experiments on a similar basis to those used for biological assay of drugs in animals, but we were limited by the fact that only two doses could be given to each patient owing to the rapid involution of the uterus and the interval necessary between doses. The assays were therefore designed as incomplete blocks of two doses. In this type of assay it is possible nevertheless to extract an activity ratio which is based only on dose contrasts within blocks, but the analysis is rather more complicated than that of an ordinary four-point assay. A comparison of ergometrine and methyl ergometrine was carried out in this way, and the analysis of variance and calculation of fiducial limits of this assay are described in detail. The calculations are adapted from Finney (1952), who discusses more complex designs. This type of design is of general interest for testing drugs on man when only two doses can be administered to each subject.

METHODS

A strain-gauge tocograph was used in these experiments which was devised and made by Dr. C. N. Smyth. The instrument consists of a brass ring, held firmly on to the abdomen by a thin elastic belt. Mounted radially within the ring is a flat strip of beryllium-copper spring, to which strain-gauges are fixed. The ebonite sensing probe passes through a collar attached to the central end of the spring. The output from the resistance bridge circuit is fed directly to a Fielden Servograph pen-recorder. The pen deflexion was proportional to the load on the spring throughout the working range.

The instrument was applied to the abdomen in the midline over the most prominent part of the surface of the uterus. The probe was adjusted to protrude against the uterus so that the load on the spring was 80 to 100 g. Each test lasted for 1 hr. For the first 20 min. the spontaneous activity, if any, of the uterus was recorded as a control period. The test dose of oxytocic was then given intravenously and its effect recorded for 40 min.

During the control period the uterus was inactive, or gave occasional intermittent contractions. Intravenous injections of small doses of ergometrine or methyl ergometrine produced either larger and more frequent intermittent contractions or sustained contractions as shown in Fig. 1. The tracings were then transcribed from circular to linear co-ordinates and the area enclosed between the base line and the tracing was used as the criterion of effect. The values obtained during the first 20 min. period after injecting the drug were used in the subsequent calculations.

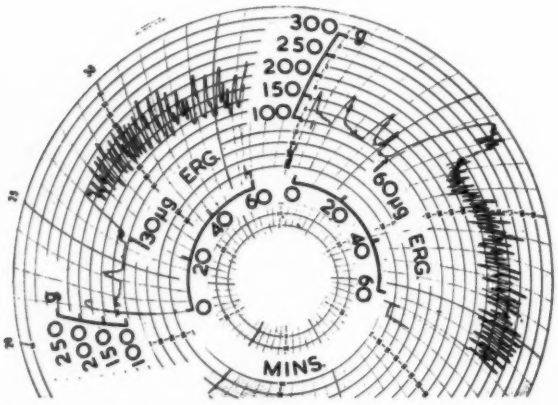

FIG. 1.—Tocograph records following intravenous injections of 30 and 60 μg. ergometrine obtained in the same subject on successive days.

Other calculations based on the whole 40 min. test period did not differ materially in the relative values obtained. Tests were made during the first four days of the puerperium, and patients of differing parity were included. Subjects with obese abdominal walls were not suitable, and, when the fundus of the uterus could not be clearly outlined by palpation, recording was not satisfactory. The nature and purpose of the tests was explained to each patient, and their consent obtained. The drugs used were ergometrine maleate and methyl ergometrine tartrate, and their activities are expressed in terms of these salts.

RESULTS

Dose/Response Curve of Ergometrine. — It seemed likely that, if several measurements could be made in the same patient, variability would be reduced particularly if these measurements could be made on the same day, since the variability due to involution of the post-partum uterus would also be thus eliminated. In a first series of experiments we therefore studied the effects of two doses of ergometrine administered to the same patient on the same day. The results of this trial are given in Table I, which shows that in each patient the first dose produced a proportionately greater effect than the second dose. We concluded that the method of giving more than one dose on the same day was unsound, since the effect of the second dose was depressed by the earlier dose.

TABLE I

EFFECTS OF TWO DOSES OF ERGOMETRINE TO THE SAME PATIENT ON THE SAME DAY

The effect is given in terms of the area between the base line and the response curve.

		Patient				Sums of Effects
		A	B	C	D	
First dose	15 µg.	13	14	12	14	53
	30 ,,					
Second dose	15 ,,	7	7	15	7	36
	30 ,,					

In the next series two doses of ergometrine were given to the same patient on consecutive days. The design was as follows: Three dose levels of ergometrine were used in all (15, 30 and 60 μg.) which had been shown in earlier trials to produce a graded response of the uterus. Each subject received two doses, arranged in such a way that every combination of doses in each order was given twice, so that 12 subjects were employed.

The results of this series are shown in Fig. 2. The pairs of doses in each subject are linked and it is seen that in each individual case the greater dose produced the greater effect. The broken line is the best fitting straight line, and shows the overall increase of response with dosage.

Certain conclusions can be drawn from these results which are relevant to the use of the method for bio-assay: (1) The basic condition of a quantitative assay, dependence of effect on dose, is fulfilled. (2) The relation between log. dose and effect is approximately linear. (3) Responses obtained in the same patient are more consistent than those obtained on different patients. These various points were borne out by an analysis of variance, which showed a highly significant slope; no significant deviation from linearity; and significantly greater variation between than within subjects.

Since the two doses were given on consecutive days it was of interest to find out whether the response of the uterus changed in a systematic

fashion during the experimental period. Our experiments are insufficient in number to establish this point with any confidence, especially since most were carried out on the second and third days and only a few were done on the first or fourth days. Nevertheless, Fig. 3 shows that there is no consistent trend in response with passage of time. This applies particularly to the response obtained on the second and third day.

Comparison of Ergometrine and Methyl Ergometrine.—This assay was also arranged in such a way that each patient received two doses of drugs. Since a 2+2 assay was planned there were six possible combinations of the four doses. Furthermore, each of these combinations could be given in the reversed order. This gives 12 possible dose combinations, or 12 patients each receiving two doses. The dose combinations were allocated to patients at random.

The results (Table II) were computed in the first instance in an approximate manner as a

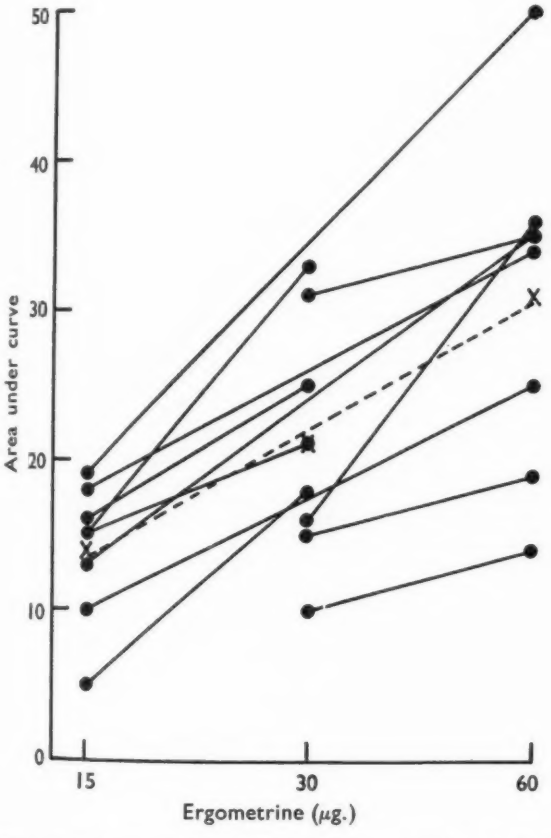

FIG. 2.—Dose/response curves of ergometrine. Points joined by a solid line refer to effects obtained in the same patient on successive days. The broken line is the best-fitting straight line.

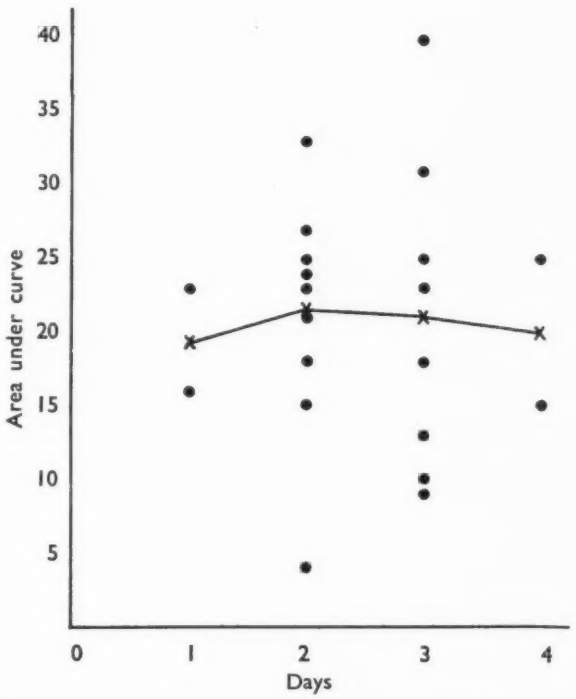

FIG. 3.—Responses to ergometrine in relation to days after delivery. (An arithmetic correction, equal to the difference in the means, was applied to equalize the three dose levels.)

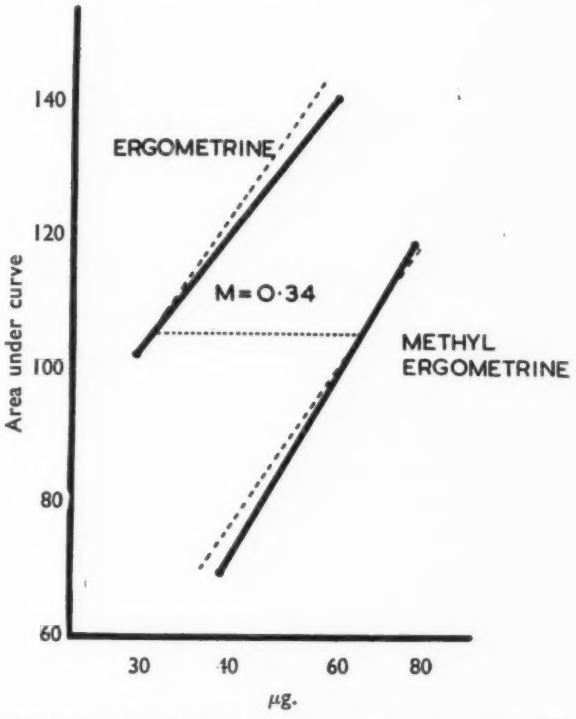

FIG. 4.—Ergometrine/methyl ergometrine assay. Crude assay results.

TABLE II

COMPARATIVE ASSAY OF ERGOMETRINE AND METHYL ERGOMETRINE

Effects measured as in Table I.

Doses	1	2	3	4	5	6	7	8	9	10	11	12
Ergometrine: 30 μg. = E_1	19	16		12			29		14	19		
60 ,, = E_2		21			23	20	40				15	25
Methyl ergometrine: 40 μg. = M_1	8		10	16				8			12	16
80 ,, = M_2			24		16	22		13	19	27		

" straight " 2 + 2 assay of 24 randomly distributed responses. The mean responses are shown in Fig. 4. The resulting activity ratio was 2.2 in favour of ergometrine and the fiducial limits were 1.3 and 11.2. (The asymmetry and wide fiducial limits are due to a large g-factor.)

The results were next computed taking into account the fact that they comprise 12 blocks of two responses with different variances within and between blocks.

The analysis of variance is divided into two sections, variation within blocks and variation between blocks. Since none of the patients received all four doses, the contrasts between doses are *partially confounded* with contrasts between patients. The object of the analysis of variance is to divide the total sum of squares in such a way that dose contrasts obtained on the same patients are separated from dose contrasts obtained on different patients.

The responses given in Table II were then used to prepare two further sets of values of the sums and the differences of the two responses for each patient. These sums and differences are multiplied by *orthogonal coefficients* as shown in Table III, to provide sums of squares for *preparations*, *regression* and *parallelism*. (The orthogonal coefficients in Table III are obtained by addition of the standard orthogonal coefficients for a four-point assay: $L_p = +1+1-1-1$; $L_i = -1+1-1+1$; $L' = -1+1+1-1$; for example, the L_p coefficient for $E_1 + E_2 = +1+1 = 2$, the L_i coeffiicent for $E_1 + E_2 = -1+1 = 0$, etc.).

TABLE III

ORTHOGONAL COEFFICIENTS FOR CONTRASTS BETWEEN AND WITHIN PATIENTS

E_1, E_2, M_1, and M_2 as in Table II.

Doses	Totals	Preparation (L_p)	Regression (L_i)	Parallelism (L')
$E_1 + E_2$	106	+2	0	0
$E_1 + M_1$	55	0	−2	0
$E_1 + M_2$	79	0	0	−2
$M_1 + M_2$	55	−2	0	0
$M_1 + E_2$	68	0	0	+2
$E_2 + M_2$	81	0	+2	0
Sum of products		102	52	−22
Divisor		32	32	32
$E_1 - E_2$	−16	0	−2	−2
$E_1 - M_1$	7	+2	0	−2
$E_1 - M_2$	−13	+2	−2	0
$M_1 - M_2$	−19	0	−2	+2
$M_1 - E_2$	−12	−2	−2	0
$E_2 - M_2$	5	+2	0	+2
Sum of products		22	120	−10
		64	64	64

Calculation of sums of squares proceeds as follows:

a. Total $Sy^2 = 9438$

b. Correction factor $\frac{(Sy)^2}{N} = 8214$

$a - b = 9438 - 8214 = 1224$

c. Sums of two doses for each patient

$[(x_1+x_2)^2+(x_3+x_4)^2 \ldots +(x_{23}+x_{24})^2]/2 = 9070$

$c - b = 856$

d. Differences of two doses for each patient

$(x_1-x_2)^2+(x_3-x_4)^2 \ldots +(x_{23}-x_{24})^2/2 = 368$

e. Between patients (from Table III)

Preparation $102^2/32 = 324$

Regression $52^2/32 = 85$

Parallelism $-22^2/32 = 15$

f. Within patients (from Table III)

Preparation $22^2/64 = 8$

Regression $120^2/64 = 225$

Parallelism $-10^2/64 = 2$

The resulting analysis of variance is shown in Table IV.

The upper part of Table IV deals with dose contrasts in different patients. It shows no significant regression and therefore provides no information about the assay. The error variance in this section is significantly greater than in the middle section, which supports the separation of the variance into two parts. The lower part of Table IV deals with dose contrasts in individual patients. It shows a significant F value for regres-

sion ($P<0.01$) and no deviation from parallelism and can therefore be utilized to provide a new activity ratio which depends entirely on measurements in individual patients and new fiducial limits.

TABLE IV
ANALYSIS OF VARIANCE

	df	Sum of Squares	Mean Square
Between patients	11	856	78
Preparation ..	1	324	324
Regression ..	1	85	85
Parallelism ..	1	15	15
Error (1) ..	8	432	54
Within patients ..	12	368	31
Preparation ..	1	8	8
Regression ..	1	225	225
Parallelism ..	1	2	2
Error (2) ..	9	133	14·8

$F = 54/14{\cdot}8 = 3{\cdot}65 \quad P<0{\cdot}05$
$F = 225/14{\cdot}8 = 15{\cdot}2 \quad P<0{\cdot}01$

The logarithmic activity ratio is given by:

$$M=\bar{x}_M-\bar{x}_E+\frac{\bar{Y}_E-\bar{Y}_M}{b}=\bar{x}\ -\bar{x}_E+\frac{Lp}{Li}\,d=0.12+\frac{22}{120}\,0.3=0.175$$

The calculation of fiducial limits for $\frac{\bar{Y}_E-\bar{Y}_M}{b}$ may be simplified by using $\frac{d}{4}=0.075$ as the dose metameter with regression coefficient b′. The fiducial limits are then given by

$$\left[\frac{\bar{Y}_E-\bar{Y}_M}{b'}\pm\frac{ts}{b'}\left\{(1-g)\left(\frac{1}{N_E}+\frac{1}{N_M}\right)+\left(\frac{\bar{Y}_E-\bar{Y}_M}{b'}\right)^2/S_{XX}\right\}^{\frac{1}{2}}\right]\div(1-g)$$

where $\frac{\bar{Y}_E-\bar{Y}_M}{b'}=\frac{Lp}{Li}\,\frac{d}{0.075}=\frac{22}{120}\,\frac{0.3}{0.075}=0.733$

$t = 2.26$ (for 9 df at the 5% level)

$s = \sqrt{14.8}=3.85$

$b' = \frac{L_i}{S_{XX}}=\frac{120}{64}$

$g = \frac{s^2t^2}{b'^2 S_{XX}}=\frac{(14.8)(2.26)^2(64)}{120^2}=0.336$

$N_E = N_M = 8$

The limits are evaluated as

$$\left[0.73\pm\frac{(2.26)(3.85)(64)}{120}\left\{(1-0.336)\left(\frac{1}{8}+\frac{1}{8}\right)+0.733^2/64\right\}^{\frac{1}{2}}\right]\div(1-0.336)$$

$= 4.023;\ -1{,}824$

These quantities must be multiplied by 0.075 in order to convert them to decimal logarithms.

$$4.023\times0.075=\ \ 0.3017$$
$$-1.824\times0.075=-0.1368$$

The lower and upper limits are therefore

$$M_U=\bar{x}_M-\bar{x}_E+0.3017=0.12+0.3017\ \ =\ 0.4217$$

$$M_L=\bar{x}_M-\bar{x}_E=0.1368=0.12-0.1368\ \ =\ 1.9832$$

Taking antilogarithms the activity ratio is 1.5 in favour of ergometrine with (5%) fiducial limits: 0.96 and 2.7. The adjusted activities as derived from within block comparisons of ergometrine and methyl ergometrine are shown graphically in Fig. 5.

DISCUSSION

These results show that it is possible to assay oxytocic drugs quantitatively on the postpartum human uterus. The external recording method involves very little discomfort for the patients, and no danger, since very small doses of drugs will stimulate the uterus when they are administered intravenously. The tocograph responses show considerable variability, but we have found that most of the variation is associated with differences between patients, presumably due to differences in the size of the uterus, its position, and the thickness of the abdominal wall. Responses obtained from the same patient were reasonably consistent, even when they were obtained on successive days. The index of precision for within-patient comparisons for the ergometrine/methyl ergometrine assay was $\lambda=0.15$, which is within the range of precision obtainable in bio-assays in animals. It is somewhat surprising that the uterus should give consistent responses in spite of involution, but in our limited series of experiments it seemed that at least between the second and third days postpartum there was no consistent diminution in response.

Since assays on the same patient are so much more efficient than on different patients, it is desirable to give more than one dose to each patient. Ideally, groups of three or four doses in each patient would be desirable, since this would give a complete assay block for each patient and provide a more efficient method of analysis, but we have found it impracticable to give three doses to each patient, and have therefore used the device of incomplete blocks with two doses only. This is less efficient than the

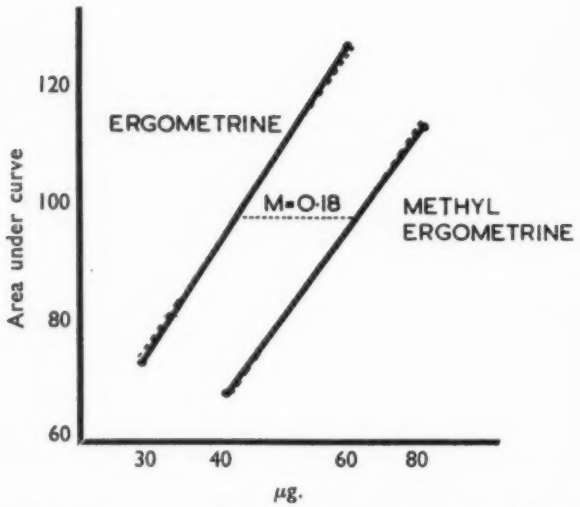

FIG. 5.—Ergometrine/methyl ergometrine assay. Adjusted values derived from comparisons in individual patients.

three doses scheme, and the analysis is more complicated, but nevertheless it is possible to obtain results with reasonable fiducial limits without using a very large number of patients.

Our finding that ergometrine is about 1.5 times as active as methyl ergometrine contrasts with the results of most other workers, who agree in claiming that methyl ergometrine is either as active as ergometrine (Kirchof, Ricely, Wilson, and David, 1944) or slightly more active than it (Gill, 1947). However, none of these comparisons were quantitative or based on concentration/action curves, and their statistical significance therefore cannot be assessed.

Although we found that both ergometrine and methyl ergometrine, when administered in small intravenous doses, gave a satisfactory dose/response relation, it does not necessarily follow that such a relation would be found with other oxytocic drugs. The effects of ergometrine and methyl ergometrine are qualitatively similar, and consist in a prolonged contraction of the uterus which can be accurately measured. Drugs which produce intermittent contraction of the uterus, such as oxytocin, are more difficult to quantitate, as suggested by the findings of Bainbridge, Nixon, Schild and Smyth (1956) with synthetic oxytocin.

We have found that the method of testing oxytocic drugs on the postpartum uterus is of value even in cases where a quantitative assessment of activity is not desired or not possible. Thus we have had occasion to test (unpublished observations) several synthetic oxytocic compounds which were sent to us by manufacturers and which had previously been tested on the animal uterus. In each case it was found that the activity of the oxytocic as compared with ergometrine was very much less on the postpartum human uterus than on the animal uterus. It would seem that for the tesing of new oxytocic drugs an assay on the intact human uterus is indispensable.

This work was carried out during the tenure by one of us (P. R. M.) of a Medical Research Council Fellowship in Clinical Research. The authors wish to thank Professor W. C. W. Nixon for access to his patients, and Dr. C. N. Smyth for the use of the tocograph.

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A COMPARISON OF SOME PHARMACOLOGICAL PROPERTIES OF DIOSCORINE AND DIOSCINE

BY

J. L. BROADBENT* AND H. SCHNIEDEN

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(RECEIVED NOVEMBER 27, 1957)

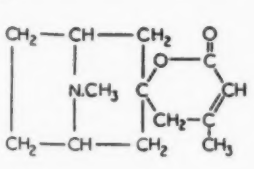
The alkaloids, dioscorine and dioscine, both obtained from yams, have been compared in respect of their convulsant activity, toxicity, analeptic action, local anaesthetic activity, adrenaline potentiating action, antidiuretic effect, and anti-acetylcholine activity. The alkaloids have similar properties, but in most respects dioscorine was the more potent. Aqueous solutions of dioscine are unstable.

The alkaloid dioscorine ($C_{13}H_{19}O_2N$) has been isolated from the tubers of *Dioscorea hirsuta* by Boorsma (1894) and the tubers of *Dioscorea hispida* by Levya and Guttierrez (1937). Dioscine ($C_{13}H_{21}O_2N$) is the name suggested for an alkaloid recently obtained from the tubers of *Dioscorea dumetorum* by Bevan, Broadbent, and Hirst (1956). In tropical lands tubers from varieties of these species are eaten. They are referred to locally as "yams." The alkaloids are of toxicological interest since poisoning characterized by convulsions may result from eating alkaloid-bearing varieties of yam.

In this paper the pharmacological properties of the two alkaloids, dioscorine and dioscine, are compared and contrasted.

MATERIALS AND METHODS

Dioscorine was supplied by Dr. A. R. Pinder, of the Department of Chemistry, University College, Cardiff, and dioscine by Professor C. W. L. Bevan, of the Department of Chemistry, University College, Ibadan, Nigeria. The chemistry of dioscine has been briefly described by Bevan *et al.* (1956), and that of dioscorine by Pinder (1952, 1953, and 1956), who proposes the following structure (Pinder, personal communication).



It has recently been reported by Bevan and Hirst (1958) that dioscine is probably an isomer of dihydrodioscorine.

The alkaloids, which were pale yellow liquids with an aromatic smell, were stored in stoppered tubes in a desiccator. Aqueous solutions of dioscorine were noticeably more opalescent than dioscine solutions of the same concentration. As dioscine is unstable in aqueous solution, freshly prepared solutions were used in the experiments except when otherwise stated.

Estimations of the intraperitoneal LD50 were made on groups of 20 white mice weighing from 18 to 26 g., the toxicity of the two alkaloids being determined simultaneously.

In studying the effects of the alkaloids on the blood pressure of the heparinized cat, the anaesthetic used was pentobarbitone sodium.

Isolated guinea-pig ileum preparations were suspended in aerated Tyrode solutions at 32°. Drugs were added directly to the bath. Local anaesthesia following intradermal injection was tested in guinea-pigs following the method of Bülbring and Wajda (1945). Antidiuretic actions were measured following the intravenous injection of the drugs into unanaesthetized hydrated male rats (weight 200 g. approximately) after the method of Ginsburg and Heller (1953). Analeptic effects were studied by measuring changes in the respiratory rate of rats anaesthetized with 60 mg./kg. of pentobarbitone sodium.

RESULTS

Convulsions.—In rats and mice both drugs caused convulsions. These were at first clonic but became tonic and death usually occurred in extensor spasm. The convulsions closely resembled those produced by picrotoxin. The LD50 value in mice was 60 mg./kg. for dioscorine and

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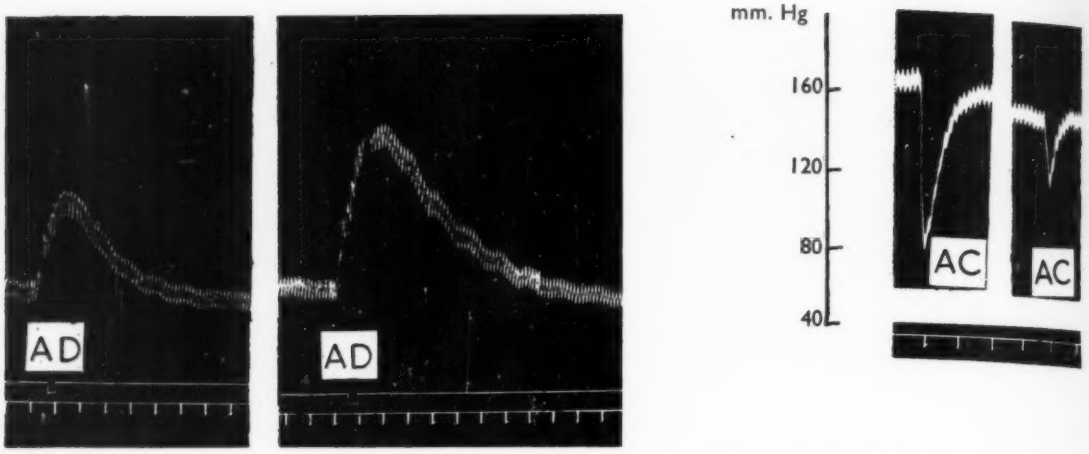

FIG. 1.—The effect of 10 μg. of adrenaline (AD) on the blood pressure of a 3 kg. spinal cat before and after 20 mg./kg. of dioscine given intravenously and the effect of 1 μg. of acetylcholine (AC) on the blood pressure of a 3.75 kg. cat before and after 15 mg./kg. of dioscine given intravenously are shown. Similar experiments have been performed thrice with dioscine and twice with dioscorine. In all experiments adrenaline actions were potentiated and acetylcholine effects were reduced. Time, 30 sec.

100 mg./kg. for dioscine. With dioscine the convulsions seemed to be at least partly spinal in origin, since spasms could be produced in spinal preparations. Dioscine convulsions in an anaesthetized cat were not attended by a measurable rise of rectal temperature or by hypoglycaemia.

Instability of Aqueous Solutions of Dioscine.—1% solutions of dioscine and dioscorine were injected intraperitoneally into mice at the estimated LD100 of 110 mg./kg. of dioscorine, and 160 mg./kg. of dioscine. Each solution killed 10/10 mice. The solutions were left overnight in a refrigerator, and tested on the following day when dioscorine killed 10/10 mice but dioscine killed only 4/10. After two days dioscorine killed 10/10 mice and dioscine 0/10 mice. When a week old, dioscorine solution killed 9/10 animals. It is thus apparent that aqueous solutions of dioscine rapidly lost convulsant properties. Anti-acetylcholine activity as measured on the isolated guinea-pig ileum was also less in old solutions of dioscine.

Analeptic Activity.—40 mg./kg. dioscorine and 60 mg./kg. dioscine, when administered intravenously, increased the respiratory rates of anaesthetized rats These doses were close to the convulsant dose.

Local Anaesthetic Effects.—A 5% solution of either dioscorine or dioscine when applied to the corneae of guinea-pigs did not prevent the corneal reflex, in fact some blepharospasm was noted. However, when injected intradermally into 12 guinea-pigs, both alkaloids had local anaesthetic activity, dioscorine in 0.5% solution and dioscine in 1% solution having approximately the same activity as 0.05% cocaine.

Effects on Cat Blood Pressure.—Neither substance produced significant changes in the cat blood pressure when injected intravenously in doses of 10 to 20 mg./kg., but the blood pressure responses to acetylcholine and adrenaline were altered. The fall in blood pressure produced by acetylcholine was reduced, and the rise in blood pressure caused by adrenaline was potentiated (Fig. 1). Mydriasis occurred during the intravenous injection of the alkaloids.

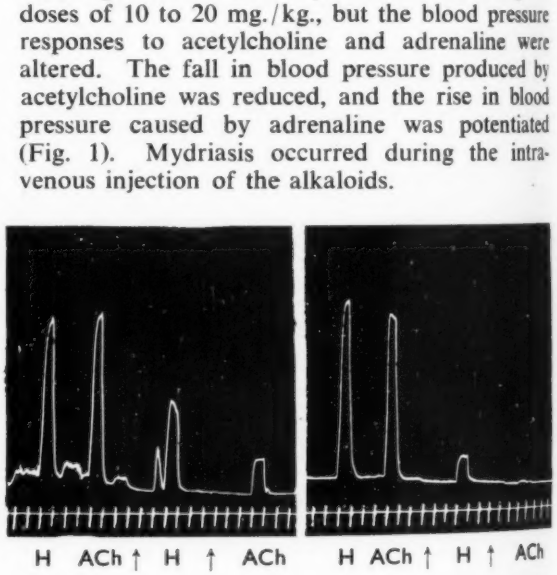

FIG. 2.—Isolated guinea-pig ileum preparation showing the effects of histamine (H) 0·5 μg. of base using the acid phosphate, and acetylcholine (ACh) 0·7 μg. The same ileum preparation was used for both tracings. The bath volume was 50 ml. The arrow in the first tracing indicates the addition to the bath of dioscine in a concentration of 10^{-5}, and in the second tracing the addition of dioscorine in the same concentration. Both alkaloids have anti-acetylcholine and antihistamine actions, but dioscorine is more potent. The experiment has been repeated several times with similar results. Time, 30 sec.

Effects on Isolated Guinea-pig Ileum.—When added to this preparation in concentrations of 10^{-5} a diminution of spontaneous movements was sometimes seen. The responses to acetylcholine and histamine were both reduced, the former more so than the latter, and dioscorine was more potent in these respects than dioscine (Fig. 2).

Dioscorine also showed slight anti-acetylcholine activity at a concentration of 10^{-6}, but dioscine was inactive at this concentration.

Isolated Rabbit Heart.—Dioscorine 2 mg. and dioscine 2 mg. had no effect on the isolated rabbit heart set up in the manner of Langendorff. But in these doses they diminished the responses of the heart to a subsequent injection of acetylcholine.

Antidiuretic Actions.—1 mg. of dioscorine had the activity of approximately 100 μU. of Pitressin, whereas 1 mg. of dioscine had the activity of approximately 20 μU. Pitressin.

Discussion

The pharmacology of dioscorine has been briefly described by Schutte (1897), Gorter (1911), and Levya and Guttierrez (1937). A more detailed account has been given by Green (1953). He described picrotoxin-like convulsions and analeptic effects and estimated that the LD50 for mice by intraperitoneal injection was 120 mg./kg., namely twice the value reported here. His observations that concentrations of 10^{-5} dioscorine did not affect the responses of isolated guinea-pig ileum to histamine and acetylcholine, and that doses of 20 mg./kg. of dioscorine did not alter the responses of the cat blood pressure to acetylcholine and adrenaline, differ from our findings.

Precise comparisons between the two alkaloids are hampered by the instability of dioscine in aqueous solutions. However, our experiments show that dioscorine and dioscine have actions that are qualitatively similar, but that dioscorine is the more toxic and has greater local anaesthetic activity, antidiuretic activity and depressant actions on the isolated guinea-pig ileum. Both resemble cocaine in causing potentiation of the pressor action of adrenaline on the cat blood pressure.

The most striking effect of the alkaloids, however, is the ability to cause convulsions, and this may be correlated with the convulsions that occur when alkaloid-bearing yams are eaten. The convulsions resemble those produced by picrotoxin and may be readily antagonized with pentobarbitone sodium (Broadbent and Reiff, 1956). Both alkaloids also resemble picrotoxin in possessing antidiuretic actions (Koiwa, 1939).

We consider that our results are consistent with the view that the two alkaloids are closely related chemically.

The authors are very grateful to Dr. A. R. Pinder and Professor C. W. L. Bevan for supplies of the alkaloids, and wish to acknowledge financial aid from the West African Drugs and Medicines Research Fund of the Nigerian Government.

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THE EFFECT OF DRUGS ON SPONTANEOUS MOTILITY AND ON RESPONSE TO STIMULATION OF THE EXTRINSIC NERVES OF THE GUT OF A TELEOSTEAN FISH

BY

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(RECEIVED JANUARY 9, 1958)

Trout gut shows, in addition to changes in general muscle tone, longitudinal " pendular " rhythms, peristaltic waves, and longitudinal " colic " contractions. Both vagus and splanchnic nerves are motor and there is no evidence of antagonistic " sympathetic " and " parasympathetic " nervous control. A posterior autonomic nerve supplying the rectum stimulated or inhibited according to the duration and frequency of the electrical pulses applied. Acetylcholine increased the tone and amplitude of pendular movements in all regions of the gut, and produced strong contraction of the circular muscles. Both nicotine and hexamethonium antagonized the action of acetylcholine, suggesting that its principal site of action is the neurone. Adrenaline lowered the tone of the longitudinal muscle and abolished pendular activity in the intestine and rectum, but contracted the longitudinal and circular muscles of the stomach. Hexamethonium bromide abolished peristalsis but greatly increased the amplitude of pendular contractions.

The reaction of the trout intestine to both histamine and pilocarpine was slight, but nicotine, 5-hydroxytryptamine, eserine, and barium caused strong contractions. The actions of atropine and piperoxan (933F) are discussed. Evidence is presented which suggests that the postganglionic vagus nerve fibres supplying the trout stomach may be adrenergic, while both the splanchnic nerve fibres to the stomach and intestine, and the preganglionic vagal fibres to the stomach, may be cholinergic. It is also suggested that there is a continuous release of acetylcholine by cholinergic neurones in the gut wall.

Anatomical studies show that the vagus nerve of teleosts is limited to the stomach, whereas the splanchnic nerve innervates both the stomach and intestine (Young, 1931; Burnstock, 1958b). Both these nerves have a motor action on the gut (Müller and Liljestrand, 1918; Young, 1936; Barrington, 1942). Acetylcholine and adrenaline, however, were reported to have antagonistic actions throughout the alimentary tract (Backman, 1917; Dreyer, 1928; Frey, 1928; Bernheim, 1934; Young, 1936). This result indicated that there was no correlation between nervous and pharmacodynamic action in the teleostean gut, a situation which has not been found in other vertebrate classes (Nicol, 1952). The question has been re-examined in the present work, using the brown trout (*Salmo trutta*). It has been shown that while acetylcholine and adrenaline are antagonistic in the intestine and rectum, they are synergistic in the stomach.

The actions of some other drugs on the intestine of a tropical marine teleost, the yellow grunt (*Haemulon flavolineatum*), were described by Bernheim (1934). Atropine antagonized the action of acetylcholine. Nicotine and barium produced strong contraction, whereas histamine and eserine had little effect. Frey (1928) and Méhes and Wolsky (1932) discussed the action of several drugs on the gut of the tench (*Tinca vulgaris*). This fish, however, is atypical of its Class since the gut contains striated muscles throughout its entire length. Both smooth and striated muscles are supplied with motor fibres from the vagus nerve.

The objects of the present study were: first, to examine the nature of autonomic nervous control of the gut in a teleostean fish; second, to compare the actions of drugs on the gut of a teleost with their actions in other vertebrates.

The brown trout (*Salmo trutta*) was used because it belongs to a teleostean family that retains many primitive and generalized features; it has not adopted a specialized feeding habit, and it is easy to keep in captivity.

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METHODS

Isolated preparations of the gut were suspended in nutrient salt solution, bubbled with 95% O_2 and 5% CO_2. Several salt solutions were tried including that recommended by Young (1932) for fresh water teleosts. A modified Krebs solution was finally chosen because it gave the strongest contractions for the longest period of time. The composition was: NaCl, 5.9; KCl, 0.25; $CaCl_2$, 0.28; $NaHCO_3$, 2.1; KH_2PO_4, 1.6; $MgSO_4 7H_2O$, 0.29; glucose 2.0, g./l.

Spontaneous pendular contractions of the longitudinal gut muscles were recorded from cylindrical segments of the stomach, intestine or rectum, suspended with open ends. Strips of longitudinal muscle were used in some experiments.

For recording circular muscle contractions, short cylinders of gut were suspended horizontally in the bath. One hook was attached to the middle of the preparation at right angles to its main axis and connected with the recording lever, while another hook held the opposite side to the bottom of the bath.

The vagus and splanchnic nerves were stimulated *in situ* electrically. In addition a nerve-muscle preparation of the stomach was used for stimulation of the vagus nerve *in vitro*.

The operculum and gills were removed from decerebrate fish to expose the vagus nerve. The pectoral girdle was dissected away. The visceral branch of the vagus was ligated near to its origin, and then dissected back to its point of entry into the oesophageal wall.

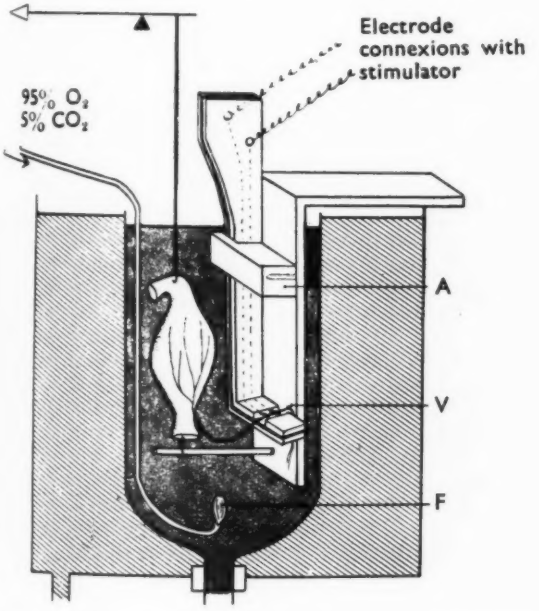

FIG. 1.—Diagram of the arrangement of the vagus-stomach preparation of the trout within the organ bath and the position of the stimulating electrodes. A, adjustable clamp; V, vagus nerve over electrodes; F, fluted glass distributor for 95% O_2 and 5% CO_2 gas mixture which is bubbled through the bath.

The stomach was severed behind the antrum and, on reflecting the ligated nerve forward, the anterior oesophagus was cut from the pharynx. The liver, swim bladder, and gonads were dissected away and the stomach with its attached nerve removed from the body cavity, after severing the other vagus nerve, the coeliacomesenteric artery and the splanchnic nerve. Connective tissue was carefully removed from the nerve trunk.

The oesophagus was tied to a perspex holder in a 100 ml. bath, while the antrum was connected to a light lever so that longitudinal contractions of the stomach were recorded. The vagus nerve was placed over bipolar platinum electrodes supported by a perspex shield. The final arrangement of the preparation in the bath is shown in Fig. 1. A rectangular pulse stimulator giving negative pulses was used to stimulate the nerve. Five second bursts of stimuli (usually of 0.3 msec. duration at 25/sec.) were applied at intervals of not less than ten minutes, so that fatigue was avoided.

The existence of a posterior autonomic nerve supplying the rectum of the trout has been demonstrated anatomically (Burnstock, 1958b). A nerve-muscle preparation was used for study of the action of this nerve on the rectum *in vitro*, using the same technique as for vagal stimulation of the stomach.

RESULTS

Spontaneous Gut Motility

Three main types of spontaneous movement were seen in the trout gut in addition to changes in general muscle tonus.

Longitudinal Pendular Contractions.—Regular contractions of the longitudinal muscle occurred approximately once every 2 min. at normal environmental temperatures (8 to 14°) (Fig. 2).

These rhythms were more marked in the empty gut, being masked or modified to a large extent by peristaltic waves in those parts of the alimentary tract swollen with food. Figs. 2*a* and 2*b* show the range of amplitudes of contraction in different intestinal preparations under identical experimental

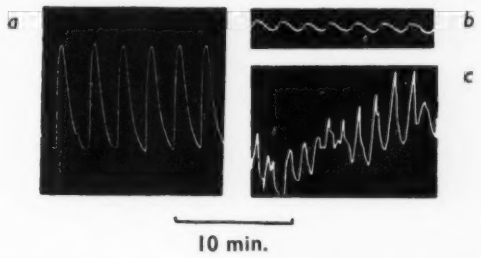

FIG. 2.—"Pendular" contractions recorded from the longitudinal muscle of the gut of the brown trout. Temperature 14°. (*a*) and (*b*), The extremes of amplitude of contractions encountered in the intestine and rectum under similar recording conditions. (*c*), Complex pendular activity often observed in the stomach.

conditions. In the stomach, pendular contractions were often more irregular (Fig. 2*c*). The rate of the pendular rhythm was very sensitive to temperature (Burnstock, 1958a) while the amplitude was affected by mechanical tension as well as temperature. No contractions corresponding to the longitudinal pendular movements were seen in the circular muscle.

Peristaltic Waves.—These occurred only when the gut was distended. The peristaltic wave moved slowly aborally (2 cm./min.) and was most prominently seen in the intestine and pyloric stomach, where it was always preceded by a powerful longitudinal contraction. Fig. 3*a* shows contraction of the longitudinal muscles superimposed over pendular activity during peristalsis. Fig. 3*b* shows contraction of the circular muscles of the stomach during peristalsis. In the cardiac stomach, antiperistalsis was commonly seen *in vivo* as well as *in vitro*.

Peristaltic waves originated in the cardia, the antrum, and in a region about one-third of the way down the intestine from the pylorus.

Longitudinal "Colic" Rhythm.—Strong, slow (1 beat/4 min.), longitudinal contractions were superimposed over the pendular activity. These contractions, like those of the circular muscle during the peristaltic wave, were triggered off by radial distension of the gut wall. They usually preceded the contractions of the circular muscles during a peristaltic wave, but this was not always so, particularly in the cardiac stomach. In the rectum, strong "colic" longitudinal contractions often occurred in the absence of circular muscle contractions (Fig. 3*c*).

No spontaneous rhythmical contractions were seen in the oesophagus. In trout with full stomachs, the circular oesophageal muscles were contracted, acting like a sphincter, preventing the regurgitation of food.

Effect of Drugs on Spontaneous Gut Motility

Acetylcholine and Adrenaline

Intestine and Rectum.—Acetylcholine (ACh) at concentrations between 10^{-4} and 10^{-8} g./ml. raised the general tone of the longitudinal muscle (Figs. 4*a* and 4*b*). The amplitude of pendular contractions was increased by concentrations of from 10^{-6} to 10^{-8} g./ml., the effect being more pronounced in preparations showing weak activity (Fig. 4*a*). Higher concentrations caused spasm during which pendular contractions were inhibited (Fig. 4*b*).

Adrenaline at concentrations between 10^{-6} and 10^{-5} g./ml. lowered the tone of the longitudinal muscle and completely inhibited pendular contractions (Fig. 4*c*).

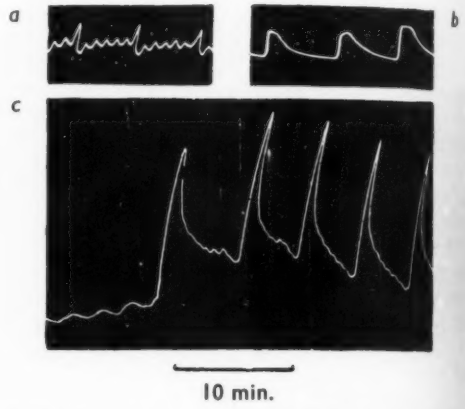

FIG. 3.—Peristalsis: 12°. (*a*), Record of the contractions of the longitudinal muscle of the intestine showing the strong peristaltic "colic" contractions superimposed over pendular activity. (*b*), The contractions of the circular muscles of the stomach during a peristaltic wave. (*c*), Longitudinal "colic" contractions, unaccompanied by peristaltic waves, as seen in the rectum. Temperature 16°

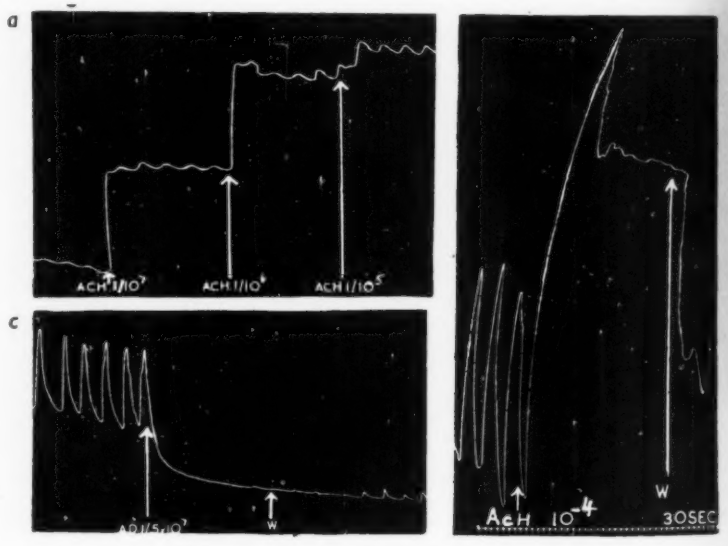

FIG. 4.—Action of acetylcholine (ACH) and adrenaline (AD) on the longitudinal contractions of the intestine. Temperature 14°. (*a*), The effect of increasing concentrations of acetylcholine on the tone and on the amplitude of pendular contractions. (*b*), The action of a high concentration (10^{-4} g./ml.) of acetylcholine. (*c*), The action of adrenaline (5×10^{-7} g./ml.). W = Wash.

Stomach.—As in the intestine, concentrations of ACh between 10^{-5} to 10^{-8} g./ml. raised the tone and increased the amplitude of the pendular contractions (Fig. 5*a*). ACh also caused strong contraction of the circular muscles, often inducing a series of regular contractions of decreasing amplitude to occur after the initial response (Fig. 5*b*). Unlike longitudinal muscle, the general tone of the circular muscle was never raised.

The reaction of the stomach to adrenaline, however, was different to that of the intestine. At concentrations of 10^{-6} to 10^{-8} g./ml., adrenaline caused a single strong contraction of the longitudinal and circular muscles, which was often followed by a depression of pendular activity (Fig. 5*b* and 5*c*). At the higher concentrations in particular, the amplitude of the pendular rhythm was decreased and there was sometimes a slight loss in general tone (Fig. 5*c*). Adrenaline applied to the stomach during an ACh-induced spasm did not cause relaxation—in contrast to the relaxation of an intestinal spasm.

On the stomach *in situ*, ACh caused longitudinal contraction and subsidiary after-contractions, whereas adrenaline produced one single contraction only. ACh and adrenaline had similar actions on the pyloric sphincter.

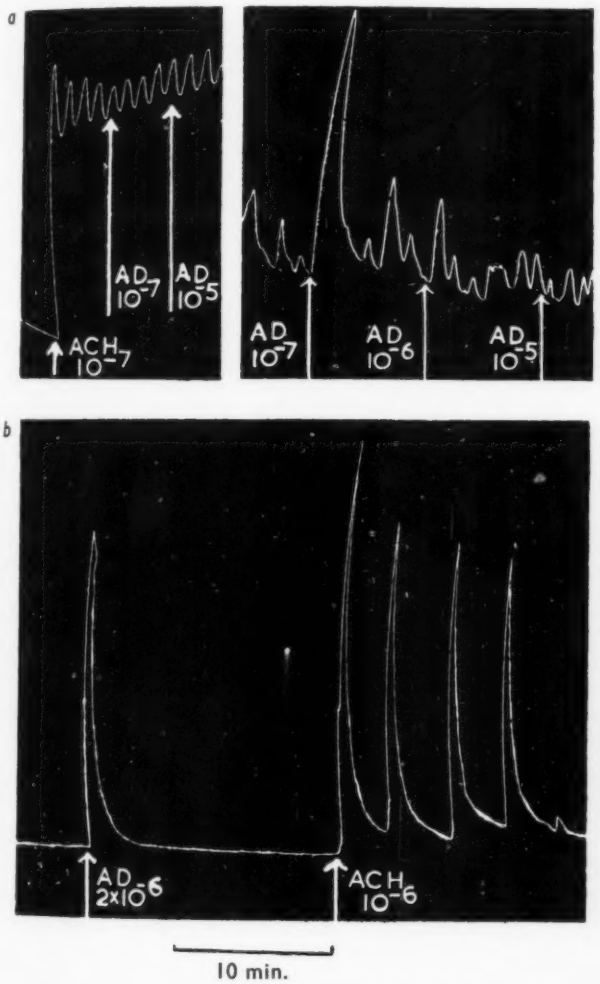

FIG. 5.—Action of acetylcholine (ACH) and adrenaline (AD) on the contractions of the circular and longitudinal muscles of the stomach. Temperature 14°. (*a*), The effect of acetylcholine (10^{-7} g./ml.) on the longitudinal muscle and the subsequent lack of response to adrenaline. (*b*), Stimulatory action of both adrenaline and acetylcholine on the circular muscles of the stomach. (*c*), The effect of adrenaline on the longitudinal muscle.

Hexamethonium

Hexamethonium bromide at concentrations of 5×10^{-4} g./ml. to 10^{-3} g./ml. stopped peristaltic activity. The action of hexamethonium on pendular contractions was very striking and was obtained consistently in over 20 experiments on the intestine and rectum, and in 10 on the stomach. At a critical concentration between 5×10^{-3} and 10^{-3} g./ml., the amplitude of the pendular contractions was greatly increased without alteration in rate (Fig. 6). This response was usually accompanied by an increase in tonus. Normal rhythm returned 15 min. after washing the

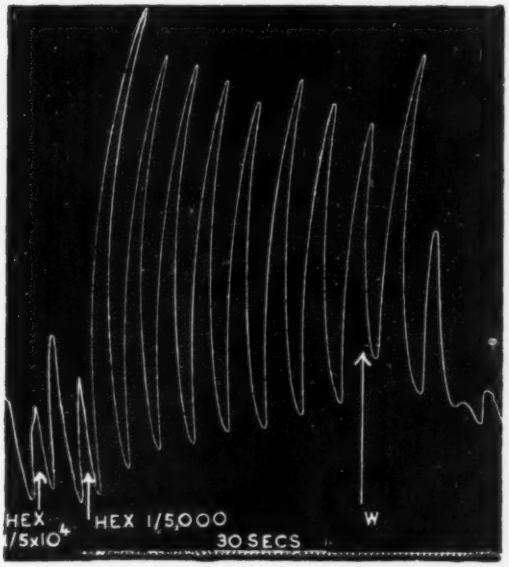

FIG. 6.—Action of hexamethonium bromide (HEX) on the longitudinal contractions of the intestine. Temperature 12°. W = Wash.

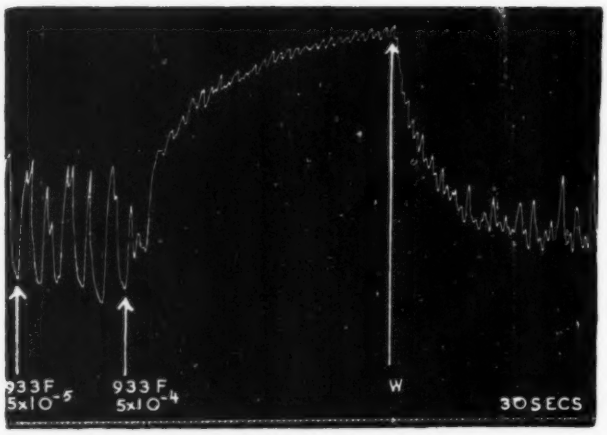

FIG. 7.—Action of piperoxane (933F) on the contractions of the longitudinal muscles of the intestine. Temperature 15°. W = Wash.

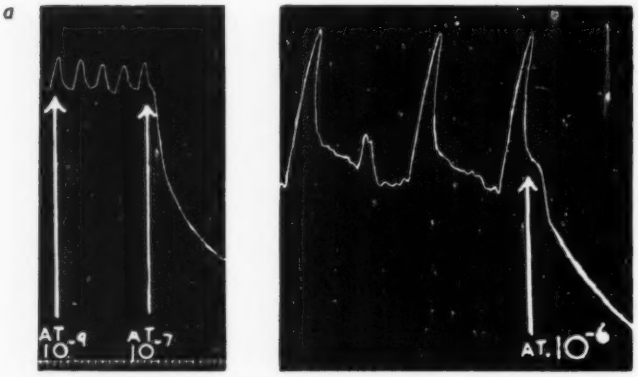

FIG. 8.—Action o f atropine (AT) on the longitudinal contractions of the intestine and rectum. Temperature 15°. Time, 30 sec. (*a*), Pendular contractions of the intestinal muscle abolished by atropine (10^{-7} g./ml.). (*b*), Colic contractions and pendular activity of the rectum abolished by atropine (10^{-6} g./ml.).

preparation. The reaction was usually more prominent in the intestine than in the stomach. The action of ACh was reduced after the application of hexamethonium.

Piperoxan (933F)

A concentration of 5×10^{-4} g./ml. increased the tone of the longitudinal muscle and decreased the amplitude of contractions both in the intestine and stomach (Fig. 7). Recovery from 933F took about 15 min. after washing the preparation with fresh Krebs solution.

Atropine

The effects of atropine were variable. Atropine at concentrations as low as 10^{-8} g./ml. decreased the tone and abolished the pendular rhythm as well as peristalsis in the intestine and rectum of 12 out of 15 preparations (Fig. 8). In the other three preparations, atropine was ineffective alone but antagonized the action of acetylcholine. In the stomach, atropine had no effect on pendular activity in 8 out of 10 preparations, but always abolished peristalsis and antagonized the action of acetylcholine.

Nicotine

Nicotine in concentrations between 10^{-7} and 10^{-4} g./ml. produced a powerful longitudinal contraction of the intestine (Fig. 9). After low concentrations of nicotine (10^{-7} g./ml.) the preparation remained sensitive to low concentrations of ACh (10^{-7} g./ml.) (Fig. 9*a*). At high concentrations (10^{-4} g./ml.), however, the response to ACh was antagonized (Fig. 9*b*).

Nicotine had no effect in the presence of hexamethonium.

Eserine

In concentrations of above 10^{-5} g./ml., eserine increased the amplitude of pendular contractions (Fig. 10*a*). There was always a latent period of 2 to 3 min. before the response, in contrast to the almost immediate reaction to ACh. Application of eserine after hexamethonium, however, failed to produce a response (Fig. 10*b*). The eserine response was also abolished as soon as hexamethonium was added to the bath (Fig. 10*c*).

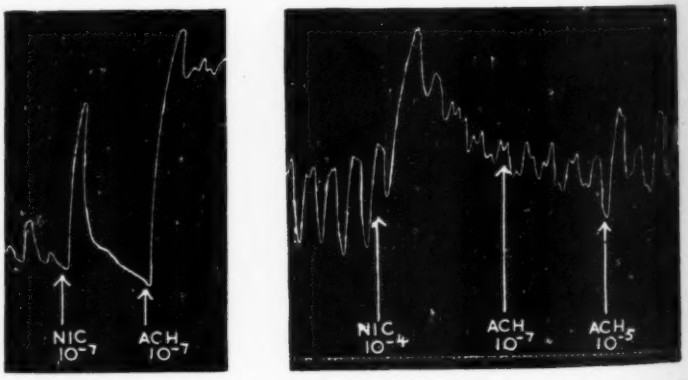

FIG. 9.—Action of nicotine (NIC) on the longitudinal contractions of the intestine. Temperature 14°. Time, 30 sec. (*a*), Effect of low concentrations of nicotine and the subsequent action of acetylcholine (ACH). (*b*), Effect of high concentration of nicotine and subsequent action of acetylcholine.

Barium Chloride

Barium chloride (10^{-3} g./ml.) caused great increase in tone and amplitude of pendular contractions in intestinal preparations. This reaction persisted, but to a slightly lesser degree, even in the presence of hexamethonium. Barium ions had little effect on stomach movements.

Histamine

Low concentrations of histamine were ineffective, but concentrations of 10^{-5} g./ml. produced a small increase in the amplitude of pendular contractions of the intestine as well as a rise in general tone. However, preparations normally at a high tonus failed to respond even to this concentration.

Pilocarpine

10^{-5} g./ml. caused a small increase in tone and amplitude of pendular contractions in the intestine, but the magnitude of the response was much less than that produced by ACh.

5-Hydroxytryptamine

Concentrations as low as 10^{-9} g./ml. caused a sustained increase in the tone of the longitudinal muscles of the stomach and intestine.

Response of the Gut to Stimulation of the Extrinsic Nerves

Fig. 11 shows the extrinsic nerves supplying the gut. The main vagus nerve trunks penetrate the wall of the oesophagus and run down the stomach, branching and anastomosing between the circular and longitudinal muscle coats. No vagal fibres extend beyond the stomach into the intestine. Stimulation of either of the vagus nerves produced contractions of the longitudinal muscles of the stomach, but had no action on the intestine or pyloric caeca. A fuller account of the form of the response and of the thresholds of stimulation has been reported elsewhere (Burnstock, 1958a).

The splanchnic nerve supplies both the stomach and intestine, branches penetrating the gut wall at intervals along its length. Stimulation of this nerve *in situ* produced contraction of both the intestine and stomach but to a lesser degree in the latter. In 5 out of 6 experiments atropine (10^{-6}) reduced this response but did not always abolish it. The existence of a posterior autonomic nerve supplying the rectum of the trout was demonstrated both anatomically and physiologically. It had a motor or inhibitory action according to the duration and frequency of the stimulating pulses applied (Fig. 12). Long duration pulses of high frequency caused inhibition of spontaneous motility, whereas pulses of short duration and low frequency stimulated the rectum.

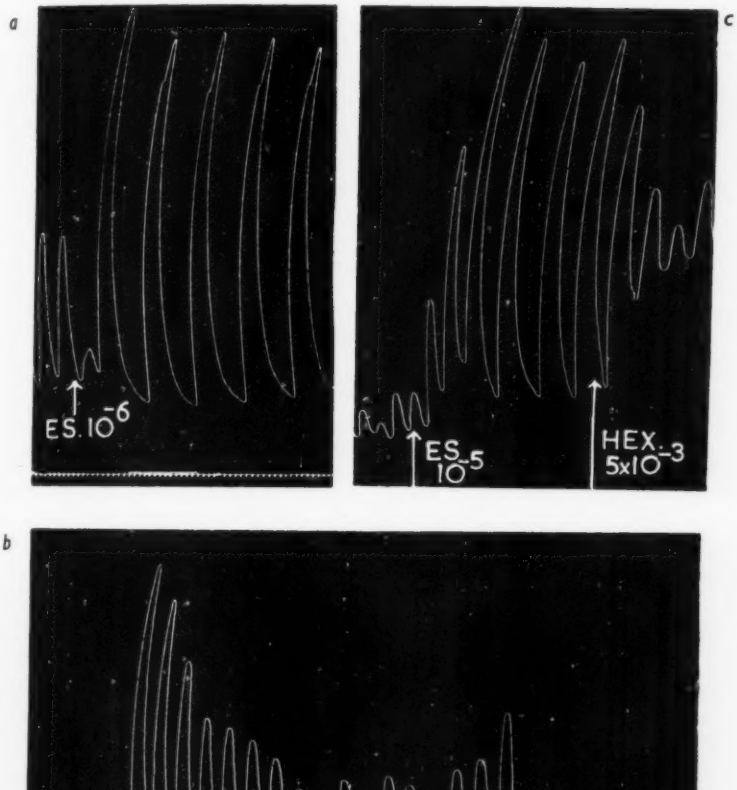

Fig. 10.—Action of eserine (ES) on the longitudinal contraction of the intestine. Temperature 14°. Time, 30 sec. W = Wash. (*a*), Effect of eserine (10^{-6} g./ml.). (*b*), Absence of the eserine action after the application of hexamethonium (HEX). (*c*), Inhibition of eserine response after the application of hexamethonium.

Effect of Drugs on the Response of the Stomach to Stimulation of the Vagus Nerves

Adrenaline

It has already been mentioned that adrenaline in low concentrations caused a contraction

of the stomach. In preparations where the response to vagal stimulation was slight, treatment with adrenaline (10^{-7} g./ml.) invariably increased the amplitude of the response (Fig. 13*a*).

Nicotine

High concentrations of nicotine (10^{-4} g./ml.) always abolished the vagal response (Fig. 13*b*).

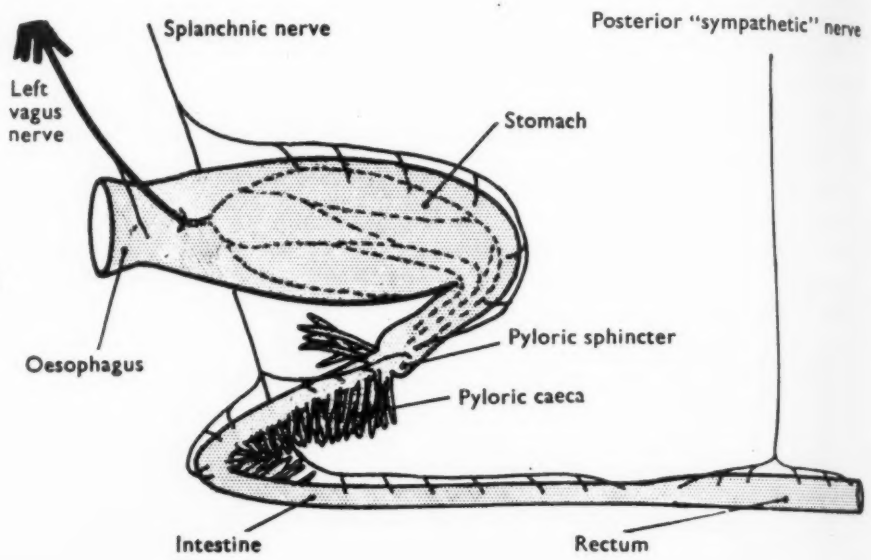

FIG. 11.—Diagram of the extrinsic innervation of the trout gut.

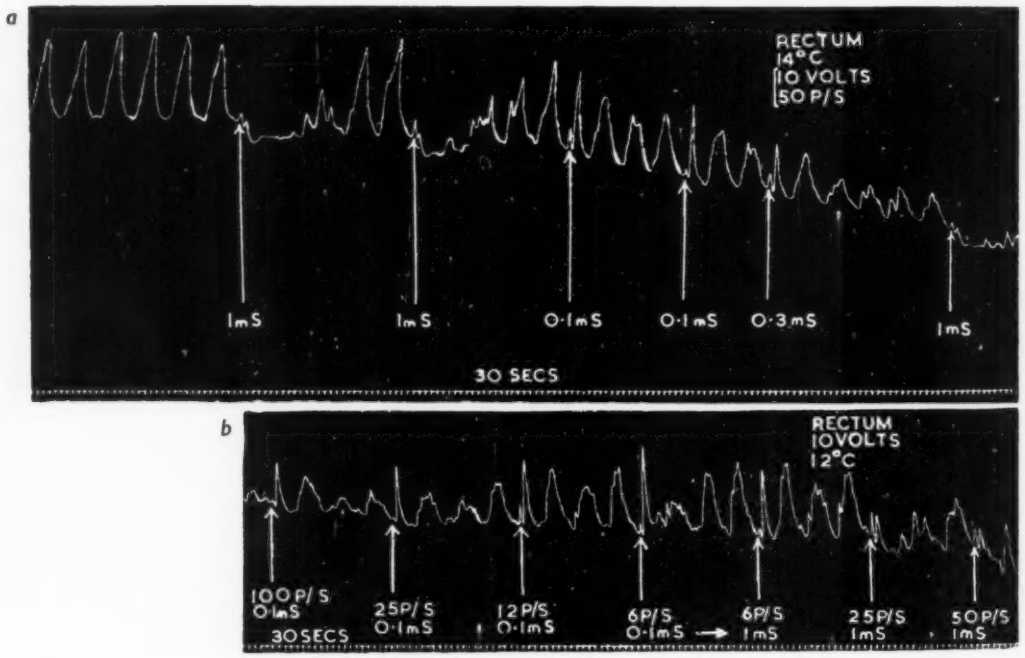

FIG. 12.—Stimulation of the posterior autonomic nerve supplying the rectum of the trout. (*a*), At a frequency of 50/sec. (P/S), pulses of long duration (1 msec.[mS]) inhibit spontaneous activity, while pulses of short duration (0.1 to 0.3 msec.) cause contraction. Temperature 14°. (*b*), Variation of the pulse frequency to show that high frequency pulses favour inhibition, while low frequency pulses cause contraction. Temperature 12°.

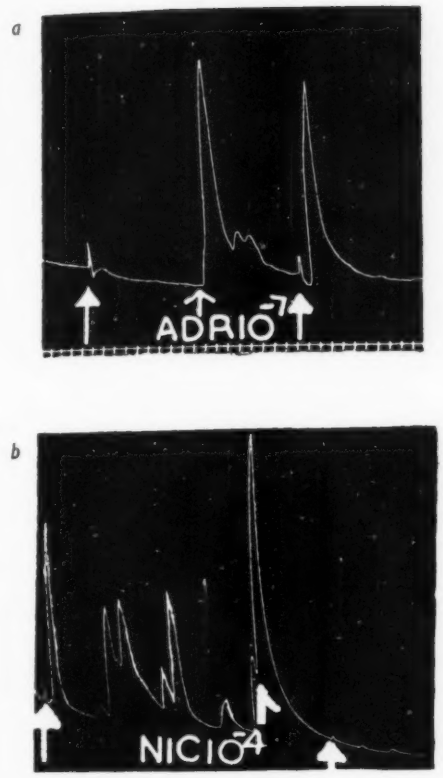

FIG. 13.—Effect of drugs on the response of the longitudinal muscles of the stomach to stimulation of the vagus nerves at arrows. Stimulus strength, 5 volts; frequency, 25 pulses/sec.; duration = 0.3 msec. Temperature 14°. Time, 30 sec. (*a*), Potentiation of the vagal response by treatment with adrenaline (ADR). (*b*), The vagal response abolished by treatment with nicotine (NIC).

Atropine

Atropine, even at high concentrations (10^{-5} g./ml.), had very little effect on the nature or magnitude of the vagal response, although the response to acetylcholine was abolished. In some experiments the amplitude of the vagal contraction was actually enhanced by atropine (Fig. 14*a*).

Hexamethonium

Concentrations of 5×10^{-3} g./ml. of hexamethonium bromide did not affect the response to vagal stimulation during the first 8 min. after its application. After this period, however, in 5 experiments out of 8, the response was abolished over the next 8 min., only to reappear in full strength after this period (Fig. 14*b*). In the other three experiments, the response was never completely lost after the application of hexamethonium.

Piperoxan (933F)

On the other hand, piperoxan, an adrenaline antagonist, when applied at concentrations of 10^{-5} g./ml., usually eliminated, and certainly reduced, the vagal response (Fig. 14*c*). Unfortunately 933F in concentrations above 10^{-5} g./ml. raised the general tone of the preparation to such a level that it cannot be certain whether failure of the vagal response was due to the drug or to the high tonus of the muscle.

DISCUSSION

Spontaneous Gut Motility

It has been shown that the spontaneous movements seen in the gut of the brown trout are similar to those in mammals—a regular "pendular" rhythm of the longitudinal muscle, peristaltic waves accompanied by powerful longitudinal "colic" contractions, and slow tonus changes. Unlike mammals, however, no myogenic rhythm occurs in the circular muscle coat, and no movements resembling "segmentation" (Cannon, 1902) have been identified in the trout gut. As the movements of isolated preparations resembled those seen *in vivo*, it seems that the extrinsic nerves only modify the intrinsic movements of the gut.

In mammals, pendular contractions occur 5 to 35 times/min., depending on the species (Garry, 1953); these are considerably higher rates than seen in the trout gut (1 every 2 min.). This difference, however, can probably be accounted for by the difference in normal environmental temperatures of the two Classes.

The pendular contractions found in the longitudinal and circular muscle coats of mammalian intestine are believed to be myogenic (Van Esveld, 1928; Feldberg and Lin, 1949; Evans and Schild, 1953; Ambache and Lessin, 1955). On the other hand, the peristaltic waves are considered to be neurogenic (Bozler, 1949; Evans and Schild, 1953; Kosterlitz, Pirie, and Robinson, 1956; Bülbring, Lin, and Schofield, 1958). Evidence is presented here that these conclusions also apply to the movements of the trout intestine. Peristaltic waves are abolished by nicotine and hexamethonium, while the "pendular" rhythm persists. Furthermore, on quickly raising the temperature of the gut, peristalsis disappears above 20° whereas the pendular rhythm persists up to 32° (Burnstock, 1958a). It has been suggested that the myenteric plexus is affected by temperature changes before the muscle cells (Ambache, 1946; Gillespie and Wishart, 1957; Innes, Kosterlitz, and Robinson, 1957; Burnstock, 1958a). It is probable, then, that the early elimination

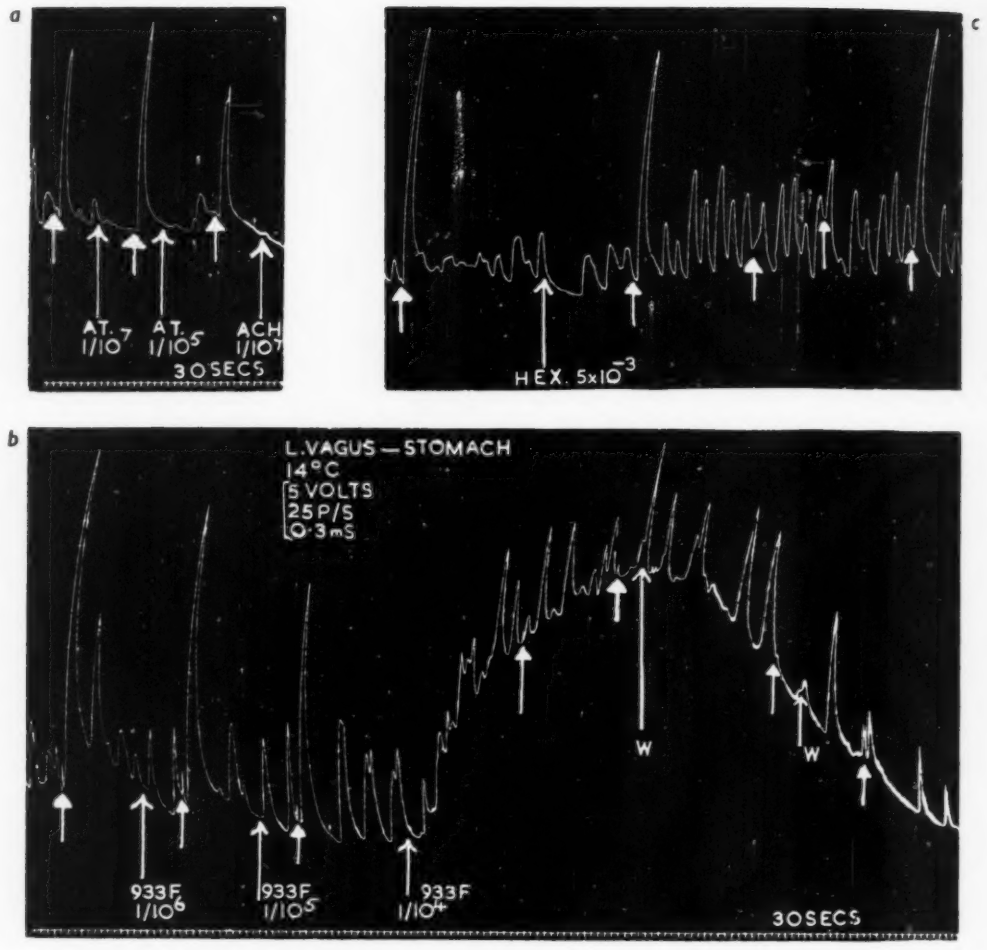

FIG. 14.—Effect of drugs (open arrows) on the response of the longitudinal muscles of the stomach to stimulation of the vagus nerves (closed arrows). Stimulus strength, 5 volts; frequency, 25 pulses sec. (P/S); pulse duration, 0.3 msec. (mS). Temperature 14°. W=Wash. (*a*), Enhancing effect of atropine (AT) on the response of the stomach to stimulation of the vagus nerves. (*b*), Effect of treatment with hexamethonium (HEX) on the vagal response. (*c*), Effect of piperoxane (933F) on the vagal response.

of peristalsis by an increase of temperature results from inactivation of nervous structures.

Since in the trout intestine the action of ACh is always excitatory, and that of adrenaline always inhibitory, it seems likely that peristaltic contractions are mediated by cholinergic neurones. Both peristaltic waves and longitudinal colic contractions are elicited by radial distension of the gut wall. A similarly triggered myenteric reflex mechanism has been analysed in mammalian gut (Kosterlitz *et al.*, 1956).

The teleostean stomach movements appear to be less complicated than those described in the mammalian stomach by Alvarez and Zimmerman (1928). The antiperistaltic wave, so characteristic in the stomach of the trout, is probably a specialized feature of this fish, since it is not prominent in other teleosts (pike, char, perch). The absence of peristaltic waves and the prominence of powerful longitudinal muscle contractions in the rectum may also be a specialized characteristic of salmonoids.

There is no previous description of teleostean spontaneous gut motility available. From the few accounts of spontaneous gut movements described in other lower vertebrates, it seems clear that peristaltic waves occur in the stomach and intestine of both amphibians (Patterson, 1928, 1933) and elasmobranchs (Young, 1933; Nicholls, 1933, 1934; Babkin, 1946). In elasmobranchs, slow tonus changes and "small contractions with a

fairly rapid rhythm " have been described (Alvarez, 1927). Presumably the latter movements can be equated with the " pendular " rhythm described in the trout gut as well as in mammals.

Eserine in concentrations of not less than 10^{-5} g./ml. markedly increases the amplitude of " pendular " contractions of the trout intestine. It is well established that eserine potentiates acetylcholine responses (Dale and Gaddum, 1930; Brown, 1937). If this is so in fish, the implication is that ACh is being continuously released in the trout's intestinal wall. The reaction of the gut to atropine supports this view. Such a mechanism has already been suggested for mammals, but, whereas Feldberg and Lin (1949, 1950) considered spontaneous release of ACh to be of non-nervous origin, both Ambache (1946) and Evans and Schild (1953) believed that postganglionic neurones in the myenteric plexus were involved. The fact that the response of the intestine to eserine is abolished by hexamethonium supports the latter view, as well as providing evidence for the nicotine-like action of ACh.

Piperoxan (933F) increases the tone of the longitudinal muscle and decreases the amplitude of pendular contractions, a response similar to the effect of high concentrations of ACh on gut movements. This implies that 933F removes an inhibitory action of adrenaline which may also be continuously released in the gut. Thus the amplitude of the myogenic pendular contractions may be controlled by a system of continuous antagonistic release of ACh and adrenaline in the gut wall. Whether the agents in such a system are of nervous or non-nervous origin remains to be seen. Burn (1950) put forward the view that adrenaline can be released locally in some organs. In mammals the existence of inhibitory adrenergic neurones in the wall of the gut has also been postulated (Ambache and Edwards, 1951).

Extrinsic Control of Gut Motility

The view that antagonistic " sympathetic " and " parasympathetic " nervous control of gut movements is absent in the lower vertebrates (Young, 1936) is supported by the present work. Both vagus and splanchnic nerves are motor to the trout gut. Moreover, the vagus nerve does not extend beyond the stomach, while the splanchnic nerve supplies the intestine as well as the stomach. It is not surprising, therefore, that the stomach and intestine often respond differently to drugs, and in particular to ACh and adrenaline, which are antagonistic in the intestine but synergistic in the stomach.

Previous workers found that ACh raised the tone and increased the amplitude of contractions in the stomach and intestine of teleosts (Backman, 1917; Dreyer, 1928; Frey, 1928; Bernheim, 1934; Young, 1936). This is also the response of the trout gut to ACh. The reaction of the trout gut to both nicotine and hexamethonium is similar to that reported in mammals (Cantoni and Eastman, 1946; Wien and Mason, 1951; Feldberg, 1951). High concentrations of nicotine or hexamethonium antagonize the response to ACh, suggesting that ACh has its principal action on the enteric neurones rather than on the neuromuscular junction or muscle fibres. Young (1936) presented evidence to support a view that the action of ACh on the bladder of the teleost *Uranoscopus* was also " nicotine-like."

Regarding the action of adrenaline there is some disagreement with previous work. Dreyer (1928), Bernheim (1934) and Young (1936) found that adrenaline caused marked loss in tone and inhibition of contractions in all regions of the gut of various marine teleosts. In the trout, this response is true for the intestine which is supplied solely by splanchnic nerves, but it is not the reaction produced in the stomach where there is vagal as well as splanchnic innervation. At low concentrations (10^{-7} to 10^{-8} g./ml.), adrenaline causes a single strong contraction of the longitudinal stomach muscles. At higher concentrations there is some decrease in the amplitude of pendular contractions, but usually only after producing a small initial contraction. These facts suggest that the postganglionic vagal motor fibres may be adrenergic, while the preganglionic fibres are cholinergic.

Some support for such a view is given by the effect of ACh and adrenaline antagonists on the vagal response. Atropine clearly does not abolish the vagal response, while piperoxan (933F), claimed to be an adrenaline antagonist in mammalian gut (Bovet and Bovet-Nitti, 1948), certainly reduces, though it does not always eliminate, electrically induced vagal contractions. In addition, pretreatment of the stomach with adrenaline usually increases the amplitude of the vagal response.

The inhibitory action of adrenaline on the intestine, together with the fact that both ACh and electrical stimulation of the splanchnic nerves produce motor responses throughout the gut, suggests that the splanchnic fibres are cholinergic. The fact that atropine reduces the effect of splanchnic stimulation supports this view.

As in teleostean fish, ACh increases the tone and contractions of the whole gut of both elasmobranch fish and amphibians (Epstein, 1931). Furthermore,

both in the stomach of elasmobranchs (Dreyer, 1928; Lutz, 1931; Nicholls, 1934; Young, 1933; Babkin, Friedman, and MacKay-Sawyer, 1935) and of amphibians (Gruber, 1923; Friedman, 1935), adrenaline in low concentrations has been reported to have a stimulating action, while at high concentrations it causes inhibition of gut movements. Hence the stimulating action of low concentrations of adrenaline on the stomach of the trout is consistent with results obtained in other lower vertebrates. In addition, the results show that there is a closer correlation between nervous action and pharmacodynamic activity than was previously demonstrated in teleosts.

This paper is part of a Ph.D. thesis, under the supervision of Professor J. Z. Young and Dr. M. E. Brown. I would like to extend my thanks to Professor J. H. Burn and Dr. Edith Bülbring for reading and criticizing the manuscript of this paper, and to the Department of Scientific and Industrial Research for providing funds for the work to be carried out.

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SUPERSENSITIVITY OF THE VESSELS OF THE PAROTID GLAND AFTER DENERVATION

BY

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Section of the postganglionic parasympathetic nerves to the parotid gland of the cat was found to cause supersensitivity of the blood vessels of the gland towards adrenaline. No supersensitivity or a slight degree only was found after cutting the preganglionic parasympathetic nerves to the gland. No supersensitivity of the vessels of the parotid gland could be demonstrated after excision of the superior cervical ganglion.

It has been found that section of the parasympathetic nerves to the parotid gland caused supersensitivity of the gland to the secretory effect of acetylcholine, pilocarpine and adrenaline (Strömblad, 1955). In accordance with the "Law of denervation" (Cannon, 1939), postganglionic denervation caused a higher degree of supersensitivity towards acetylcholine and pilocarpine than preganglionic denervation. The sensitivity to adrenaline was, however, not greater after postganglionic than after preganglionic denervation. It was thought that this discrepancy could be due to vascular changes caused by adrenaline. If the vessels obeyed the law of denervation, vasoconstriction might be greater in the postganglionically denervated than in the preganglionically denervated gland and thus the secretory effect would be restricted more in the former case. This has now been investigated further.

It is generally assumed that the parasympathetic nerves to the parotid gland of the cat contain vasodilator fibres and that the sympathetic nerve contains vasoconstrictor fibres. Evidence is, however, scanty and it therefore seemed of interest to make further observations on the effects of stimulation of these nerves.

METHODS

Cats were anaesthetized with chloralose (80 mg./kg. body weight). The parotid region was exposed on both sides; cannulae were tied into the posterior facial veins and branches of these veins, other than those coming from the parotid glands, were ligated. Heparin was used to prevent clotting. The drops of blood falling from the cannulae were signalled manually to ordinate recorders (Clementz and Ryberg, 1949). In most experiments cannulae were also introduced into the parotid ducts.

Injections were made through a cannula inserted into the femoral vein and blood collected from the parotid gland was returned to the animal from time to time by the same route. The blood pressure was recorded from the femoral artery. In some animals parasympathetic denervation of the parotid gland on one side was done by the methods previously described (Strömblad, 1955); in others the superior cervical ganglion was excised on one side. These operations were done two weeks before the acute experiment.

RESULTS

Blood Flow Through the Parotid Gland During Stimulation of Parasympathetic and Sympathetic Nerves

Fig. 1 shows the vasodilator effect of stimulation of Jacobson's anastomosis (preganglionic parasympathetic). There were often two phases of increased blood flow separated by a short period during which the flow was somewhat less. This was especially marked in the case shown in the left-hand record where the **W**-type response can be clearly seen. After atropine, vasodilatation still takes place but secretion is abolished. This atropine-resistant vasodilatation, similar to that in the submaxillary gland, was observed by Langley (1889), who, however, remarked that the flushing of the gland was less marked than he had expected. In our experiments the vasodilatation after a moderate dose of atropine (1 mg.) was usually as pronounced as without atropine in spite of the fact that atropine had completely suppressed the secretion. After atropine the **W**-shape of the response was not evident. Large doses of

atropine (8 to 10 mg.) lessened the vasodilatation caused by stimulation of the parasympathetic nerves though they did not abolish it.

Stimulation of the sympathetic nerve in the neck caused a pronounced decrease in the blood flow through the gland (Fig. 2). This decrease was maintained for as long as the stimulation was continued and was not interrupted by periods of flow greater than the resting flow, as is seen with the submaxillary gland. Even during intense vasoconstriction the gland was still able to secrete. In one case, for example, 7 drops were obtained during 5 min. of stimulation. Cessation of stimulation of the sympathetic was always followed by a period of increased flow through the gland.

Effect of the Injection of Adrenaline on the Blood Flow Through the Parotid Gland

The intravenous injection of adrenaline caused moderate changes in the flow of blood through the normal gland (Fig. 3, lower record). In some experiments there was an increase in flow coinciding with the increase in blood pressure while in others there was no change or a slight decrease. It was often found that, in the same animal, small doses (1 to 10 μg./kg.) caused an increase and larger doses a decrease in flow. Very large doses (100 to 200 μg./kg.) regularly caused a decrease. In most animals, after the blood pressure had returned to its original value, there was a period during which the flow was slightly diminished.

Postganglionic Parasympathetic Denervation.—On the denervated side, adrenaline always caused a pronounced fall in blood flow through the gland. In Fig. 3 the effects caused in the normally innervated gland (lower records) and in the contralateral denervated gland (upper records) can be compared. In the denervated gland, the decrease in flow both during the phase of increased blood pressure and in the following period is much greater. Even in cats in which an injection of adrenaline caused an increased flow through the control gland, there was a decreased flow through the denervated gland.

The changes after denervation described above were found in 9 out of 11 cats used. In one of the remaining two cats, denervation was found to

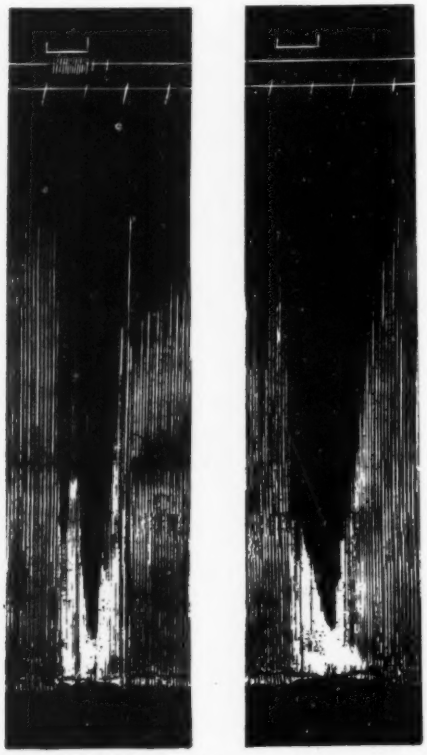

FIG. 1.—Effect of stimulation of Jacobson's anastomosis (preganglionic parasympathetic) on blood flow through the parotid gland. Cat 2·9 kg. Tracings from above downwards: signal; drops of saliva; time in min.; blood flow. An increase in the height of the lowest trace indicates a reduction in blood flow. Atropine (1 mg.) was injected during the interval between the two tracings.

FIG. 2.—Effect of stimulation of the sympathetic nerve in the neck on blood flow through the parotid gland. Cat 2·8 kg. Tracings from above: signal, time in min., drops of saliva and blood flow.

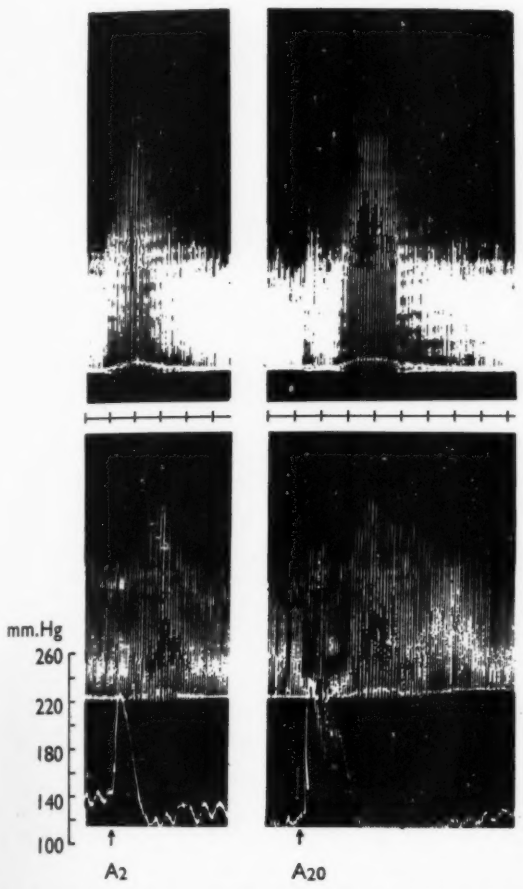

FIG. 3.—Effect of adrenaline on blood flow in the postganglionically denervated (upper) and the contralateral normally innervated gland (lower). Cat 3·1 kg. Time, in min. between upper and lower tracing. A_2 and A_{20}, 2 and 20 μg./kg. of adrenaline, respectively.

have been incomplete: preganglionic stimulation evoked secretion. In the other, the success of the operation was unfortunately not tested. Acute denervation of the gland was never followed by the changes described above. In the resting state, the flow of blood was not appreciably different through the previously denervated and through the normal gland.

Preganglionic Parasympathetic Denervation.—In 5 out of 7 cats used, the vascular response to adrenaline was the same on the innervated and denervated sides, and there was no difference in the resting flow. It was found in the remaining two animals that 20 to 50 μg./kg. of adrenaline caused a decrease in the flow of blood on the denervated but not on the control side. On further increasing the dose to 100 μg./kg., a decreased flow was found on the control side as well. No difference could be seen after small doses of adrenaline. It may be concluded from these experiments that preganglionic parasympathetic denervation causes no or only a slight degree of supersensitivity of the parotid vessels.

Excision of the Superior Cervical Ganglion.—In both acute and chronically operated animals the flow through the gland in the resting state was about twice as much on the operated as on the unoperated side. There was no difference in the vascular response to adrenaline on the two sides, but the difference in resting flow could have affected this result and a change in sensitivity cannot be excluded.

DISCUSSION

The difference in the effect of adrenaline on the flow of blood through the normally innervated and postganglionically denervated gland may well be explained by the development of a supersensitivity in the vessels of the denervated gland.

Supersensitivity of blood vessels has been shown to follow sympathetic denervation in various vascular regions, but in some cases none has been found (see Cannon and Rosenblueth, 1949; Griffin, Green, Youmans and Johnson, 1954; Burn, Philpot and Trendelenberg, 1954). We would not exclude, on the basis of the present experiments, the possibility that sympathetic denervation of the parotid vessels may cause supersensitivity, but the experiments do not support the idea. On the other hand, they do show that postganglionic parasympathetic denervation causes supersensitivity of the vessels, and so far as we know it is the first time that this has been demonstrated. No supersensitivity or a slight degree only was found after preganglionic parasympathetic denervation. Thus, the vessels of the parotid gland are another example of the superiority of postganglionic, as opposed to preganglionic, denervation in causing supersensitivity.

In earlier experiments, it was found that the supersensitivity to the secretory action of adrenaline after preganglionic denervation was of the same magnitude as after postganglionic denervation, and the suggestion was put forward that this was due to the higher sensitivity to adrenaline of the blood vessels after postganglionic denervation. This suggestion is supported by the results obtained in the present series of experiments. It is therefore possible that the secretory response was restricted by the vascular reactions in the postganglionically denervated parotid gland.

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LOCAL ACTION OF SOME ANTIBACTERIAL SUBSTANCES AGAINST *CORYNEBACTERIUM OVIS* IN GUINEA-PIG SKIN

BY

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When a suspension of living *Corynebacterium ovis* was injected intradermally in guinea-pigs, a lesion of roughly circular outline developed within 24 hr. Lesions of smaller diameter arose if benzylpenicillin, dequalinium, hedaquinium, cetrimide, or oxytetracycline were injected at the identical site, either before, with, or after *C. ovis*. Evidence has been obtained that such reductions of lesion diameter are due to direct action of drugs on bacteria and not to antitoxic or anti-inflammatory actions. Lesion diameters became less as drug dosage increased up to a limit, and these reductions provided a measure of local antibacterial action *in vivo*. Intradermal injection of higher concentrations of antibacterials, without *C. ovis*, produced comparable but somewhat flatter lesions, diameters of which increased with increasing concentration of drug and provided a measure of intradermal toxicity.

In clinical practice, bacterial infections of skin and mucous membranes are often treated by local application of antibacterial substances. To evaluate the topical efficiency of new antibacterials, we have attempted to develop a method in guinea-pigs, based on the immunological work of Miles (1949), in which bacteria and drug are injected intradermally at the same site.

Miles (1956) and Miles, Miles, and Burke (1957) have shown that several species of bacteria produce measurable lesions when injected intradermally in guinea-pigs. For the work described in this paper, we chose *Corynebacterium ovis* for several reasons. First, the lesions produced were large, regular and clearly marked. Second, this species was not only sensitive to the drugs which we wished to evaluate, but to our reference compound penicillin, which has been much used topically in clinical practice. Third, Miles had shown that *C. ovis* lesions in guinea-pigs were particularly easy to reduce by the systemic administration of penicillin. This suggested that local chemotherapeutic effects might also be expected.

MATERIALS AND METHODS

Drugs

We used dequalinium chloride (decamethylenebis-(4-aminoquinaldinium chloride), Dequadin), hedaquinium chloride (hexadecamethylenebis-(2-*iso*quinolinium chloride), Teoquil), benzylpenicillin (potassium salt) B.P., procaine penicillin B.P., oxytetracycline hydrochloride B.P., cetrimide B.P. and cortisone acetate B.P. Weights of drugs are expressed throughout as salts.

Inocula

C. ovis (B1/50) was maintained on Löffler slopes. In preparing inocula for *in vitro* tests, cultures were grown at 37° for 18 hr. in 1% peptone-water containing 10% normal horse serum (serum-peptone), diluted in the same medium to contain *circa* 10^7 organisms/ml. (opacity 2, Brown's tube), and then by 1/100. For intradermal injection, the 18 hr. growth from a Löffler slope was suspended in 0.9% NaCl in water (saline) and diluted in saline to opacity 2. The *in vivo* inoculum was prepared by diluting this suspension as required.

In some *in vivo* experiments inocula were treated either by exposure to boiling water for 30 sec. or by incubation with potassium penicillin at a concentration of 10 μg./ml. for 18 hr. at 37°. The action of penicillin was terminated by adding penicillinase from *Bacillus subtilis* and standing for 30 min. at room temperature. Destruction of penicillin by penicillinase was confirmed by plate assay. Sterility tests on treated cultures were performed in serum-peptone.

Experiments in vitro

An inoculum of 0.02 ml. was added to each 5 ml. aliquot of series of geometrically decreasing concentrations of each antibacterial in serum-peptone. Tubes were incubated at 37° and examined visually for growth at 1 and 5 days. The minimal inhibitory concentration (MIC) was taken as the lowest concentration of drug inhibiting visible growth for each period.

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Experiments in vivo

Before intradermal injection, guinea-pigs were shaved with an electric razor and then depilated chemically (Sleek, Elizabeth Arden). Intradermal injections were carried out with short bevel, 0.45 mm. needles. In order to make a second injection along the same needle path as the first (superinjection), the needle tip was dipped in Indian ink before being inserted the first time (Miles, 1949). Doses are expressed throughout as quantities injected/site. Drugs were administered, in geometrically decreasing concentrations, either dissolved or suspended in saline.

Generally 4 guinea-pigs were used in each test; and in all experiments, where appropriate, drugs, bacteria and/or saline were injected intradermally in each guinea-pig at 16 sites, doses being arranged in a 4 × 4 Latin square. All lesions were roughly circular in outline. An ink line was drawn around the edge of the lesion and two diameters at right angles measured with spring-bow dividers against a Vernier scale. The arithmetic mean of these diameters was taken as a measure of response at each site and is referred to below as lesion diameter.

With C. ovis.—In tests with *C. ovis*, guinea-pigs weighing 200 to 250 g. were used. In some tests (simultaneous), equal volumes of drug and of inoculum were mixed and immediately afterwards 0.1 ml. of the mixture was injected at each site. In prophylactic tests, 0.05 ml. of the drug preparation was injected and later 0.05 ml. of the inoculum superinjected. This order of administration was reversed in therapeutic tests. For control purposes, *C. ovis* without drug was given routinely, either in a single injection of 0.1 ml. or in 0.05 ml. preceded or followed by 0.05 ml. saline. As an additional control, saline was given either in 0.1 ml. or in 0.05+0.05 ml. injections.

Diphtheria Toxin.—Schick test toxin B.P., diluted 1/6, was mixed with an equal volume of saline, or of dequalinium or penicillin in saline, and 0.1 ml. aliquots were injected intradermally in guinea-pigs weighing 200 to 250 g. Schick control B.P. was similarly diluted, mixed with saline and injected in the control sites.

Tuberculin.—About 5 weeks before giving purified protein derivative of old tuberculin B.P., guinea-pigs weighing 350 to 400 g. were sensitized by subcutaneous injection of 0.5 ml. of suspension containing 2 mg. of killed *Mycobacterium tuberculosis*. In some experiments, three successive daily subcutaneous doses each of 1 mg. cortisone in 0.5 ml. saline were given and, 4 hr. after the last dose, 0.2 ml. of tuberculin in saline was injected intradermally. In other experiments, guinea-pigs received intradermal doses of cortisone, dequalinium or hedaquinium, and, 30 min. later, 0.1 ml. of tuberculin in saline was superinjected.

Results

Experiments in vitro

The geometric mean MIC values obtained with the antibacterial substances used are given in Table I.

TABLE I

IN VITRO ACTIVITIES OF SOME ANTIBACTERIAL SUBSTANCES AGAINST *CORYNEBACTERIUM OVIS* IN SERUM-PEPTONE WATER AT 37°

MIC refers to minimal inhibitory concentration.

Drug	Geometric Mean MIC (μg./ml.)	
	1 Day	5 Days
Dequalinium chloride ..	0·110	0·312
Hedaquinium chloride ..	0·221	0·221
Benzylpenicillin	0·039	0·055
Oxytetracycline hydrochloride	0·276	3·125
Cetrimide	4·416	4·416
Procaine penicillin	0·110	0·156

It will be seen that all drugs readily inhibited *C. ovis* in serum-peptone.

Intradermal Experiments

Saline

Twenty-four hr. after saline had been given at one site, either in a single injection of 0.1 ml. or in two of 0.05 ml., we regularly observed a very small flat lesion, presumably due to mechanical damage. This mark may just be discerned in Fig. 1. Throughout the whole series of tests, the diameter of this

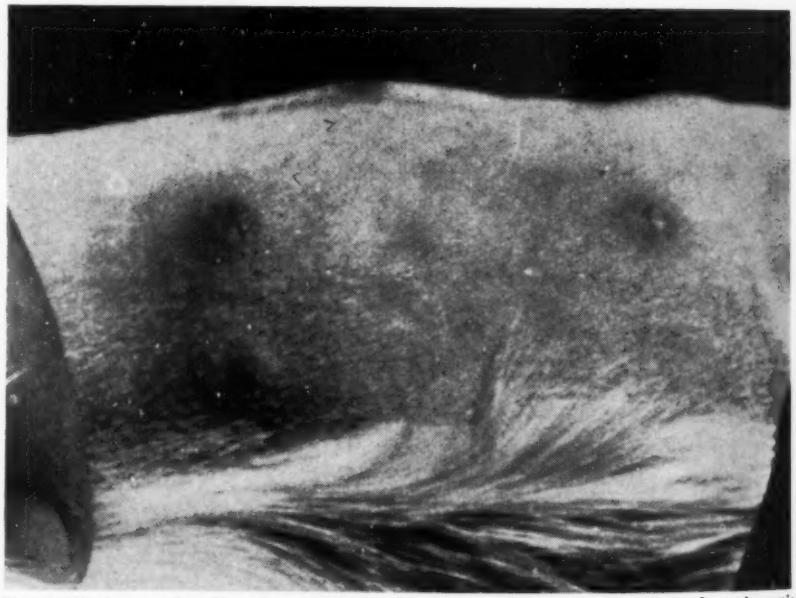

FIG. 1.—Lesions produced in guinea-pig skin by intradermal injection of 0.1 ml. *Corynebacterium ovis* suspensions 24 hr. previously. Upper row (left to right): opacity 2; opacity 2 boiled for 30 sec.; opacity 2/100. Lower row (left to right): opacity 2/10; no injection; 0.1 ml. 0.9% NaCl in water.

lesion did not diverge appreciably from the overall mean of 1.925 mm., whether the saline was given in single or divided doses.

C. ovis *without Drugs*

Living Bacteria.—Injection with *C. ovis* produced at 24 hr. a bright red conical lesion (Fig. 1), the outline of which was clear in the living animal. To examine the effects of inoculum density and time of measurement, 4 guinea-pigs were injected with *C. ovis* suspensions at opacities 2, 2/10, and 2/100. At the control sites, one animal received saline, a second boiled inoculum at opacity 2, and the other

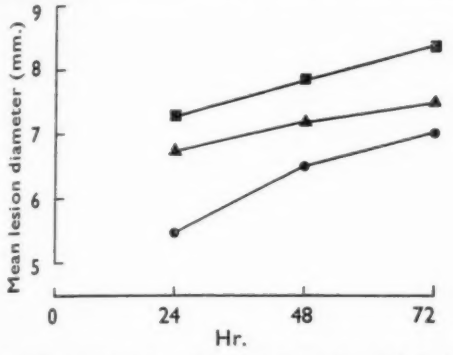

FIG. 2.—Effects of inoculum density and duration of infection on diameter of lesions due to intradermal injection of 0.1 ml. *Corynebacterium ovis* in guinea-pigs. Points at 24 hr. are based on 4 sites in each of 4 animals, subsequent points on 4 sites in each of 3 animals. ■, opacity 2 (Brown's tube); ▲, opacity 2/10; ●, opacity 2/100.

two boiled inoculum at opacity 2/10. The guinea-pig receiving saline died between 24 and 48 hr. after inoculation. All lesion diameters in all animals were measured at 24 hr. and in the survivors at 48 and 72 hr. The mean lesion diameters for the 16 and later 12 sites inoculated with living *C. ovis* are plotted as the ordinate against time in Fig. 2. It will be seen that lesion diameter increased with inoculum density and with time.

As by 48 hr. one pig had died and lesion edges in survivors were harder to distinguish, we chose 24 hr. as the time of measurement in subsequent experiments. In these we used an inoculum corresponding to 0.1 ml. at opacity 2/10 (0.05 ml. at opacity 2/5). At this inoculum density, no deaths occurred within 24 hr., confirming the general finding of Miles *et al.* (1957).

Bacteria Exposed to Heat or Benzylpenicillin.—In the experiments described above, boiled inocula at opacity 2 or 2/10 produced no larger lesion at 24 hr. than did saline (see Fig. 1).

In other experiments, cultures exposed to penicillin failed to produce within 24 hr. lesions larger than those produced by saline or by penicillin+penicillinase without bacteria.

Sterility tests showed that no bacteria survived being boiled for 30 sec.; but, after exposure to penicillin, some growth occurred after 4 days in serum-peptone, presumably due to a few surviving bacteria. The guinea-pigs inoculated with penicillin-treated cultures were kept and small lesions also appeared after 4 days.

C. ovis *with Drugs*

Antibacterials.—Injecting any of the antibacterial substances in suitable doses before, with or after *C. ovis* at the same site produced lesions smaller in diameter than those arising from *C. ovis* alone. This result is exemplified by the experiment illustrated in Fig. 3, in which dequalinium solutions were superinjected 30 min. after *C. ovis*.

In order to examine the effect upon lesion diameter of the time interval between injections of bacteria

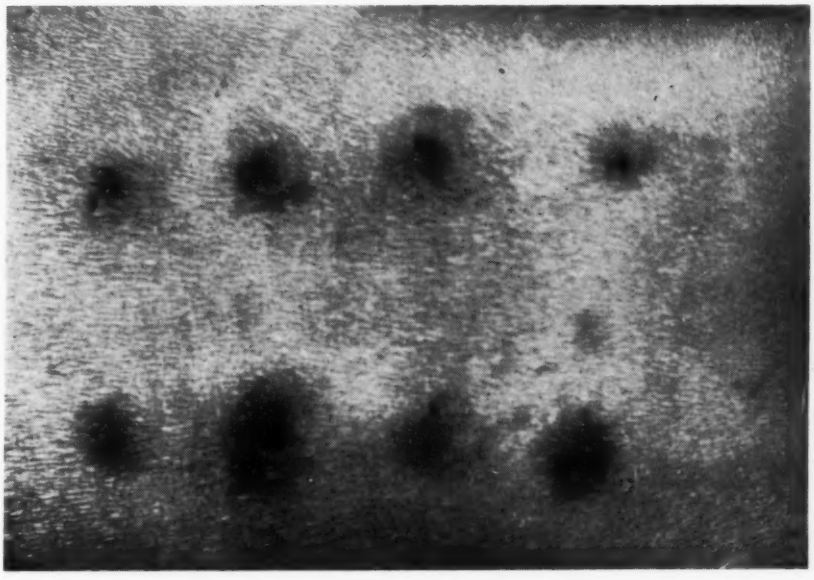

FIG. 3.—Lesions produced in guinea-pig skin by intradermal injection of *C. ovis* suspension followed by dequalinium solution 30 min. later along the same needle path. Upper row (left to right): *C. ovis* +0.2 μg. dequalinium; *C. ovis* +0.05 μg. dequalinium; *C. ovis* alone; *C. ovis*+0.8 μg. dequalinium. Lower row (left to right): *C. ovis*+0.05 μg. dequalinium; *C. ovis* alone; *C. ovis* + 0.8 μg. dequalinium; *C. ovis*+0.2 μg. dequalinium.

TABLE II

EFFECT ON LESION DIAMETER OF TIME INTERVAL BETWEEN INTRADERMAL INJECTIONS OF *C. OVIS* AND OF BENZYLPENICILLIN ALONG THE SAME NEEDLE PATH IN GUINEA-PIGS

S.E. refers to standard error of the mean based on diameters of 16 lesions.

Expt. No.	Treatment	Mean Lesion Diameter ±S.E. (mm.)
1	*C. ovis* alone	5·638±0·105
	Drug 2 hr. before *C. ovis*	4·730±0·079
	,, 1 ,, ,, ,,	4·670±0·057
	,, 0·5 ,, ,, ,,	4·544±0·066
2	*C. ovis* alone	5·972±0·141
	Drug 0·5 hr. after *C. ovis*	5·020±0·083
	,, 1 ,, ,, ,,	5·124±0·100
	,, 2 ,, ,, ,,	5·075±0·108

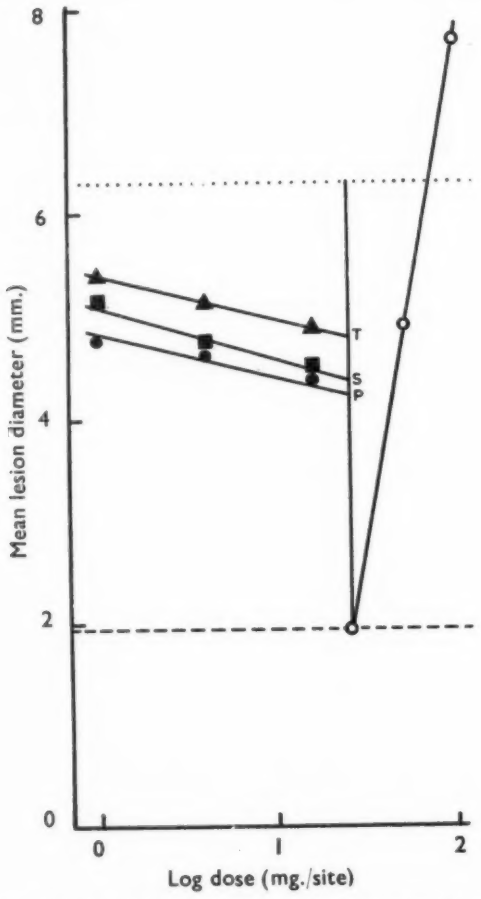

FIG. 4.—Relation of intradermal dose of benzylpenicillin in guinea-pigs to lesion diameter in the presence and absence of *C. ovis*. In antibacterial tests, 0.05 ml. benzylpenicillin solution + 0.05 ml. *C. ovis* suspension were injected at the same site. In toxicity tests 0.1 ml. benzylpenicillin solution was injected. ●, Benzylpenicillin 30 min. before *C. ovis*; ■, benzylpenicillin with *C. ovis*; ▲, benzylpenicillin 30 min. after *C. ovis*; ○, benzylpenicillin alone; . . . . , diameter of lesion produced by *C. ovis* alone; – – –, diameter of lesion produced by 0.05 + 0.05 ml. 0.9% NaCl in water. For explanation of P, S, and T, see discussion.

and of drug, benzylpenicillin was given at various times before *C. ovis* in one experiment, and after *C. ovis* in another. The mean lesion diameters obtained in both experiments are given in Table II.

Since it was inconvenient to superinject at an interval shorter than 30 min. and since longer periods of up to 2 hr. between injection and superinjection gave no greater reduction, we adopted a 30 min. time interval in all prophylactic and therapeutic tests.

The results of a series of experiments, in which benzylpenicillin was given at three dose levels in prophylactic, simultaneous and therapeutic tests are expressed in the left-hand portion of Fig. 4, in which lesion diameters are plotted as ordinates against log dose. The horizontal dotted line represents the mean diameter over this series of tests with benzylpenicillin of the lesions caused by *C. ovis* alone, and the horizontal broken line the mean diameter of the lesions due to saline. In Fig. 5, results obtained with hedaquinium are expressed in the same way.

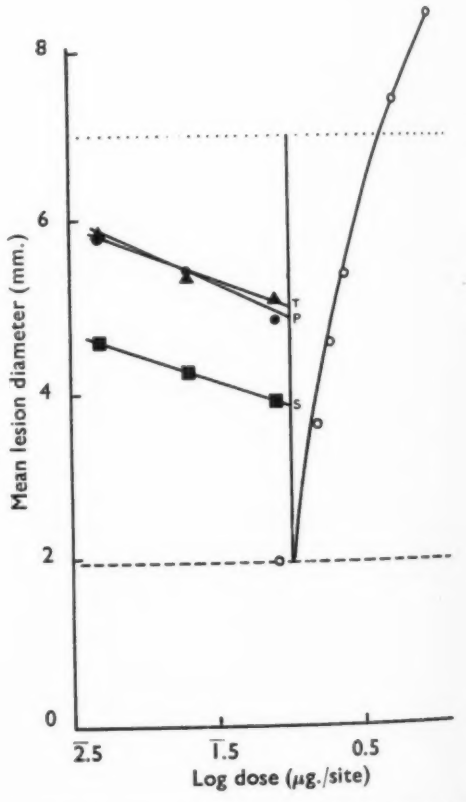

FIG. 5.—Relation of intradermal dose of hedaquinium in guinea-pigs to lesion diameter in the presence and absence of *C. ovis*. Method of test and symbols as in Fig. 4.

A similar graph was constructed for each drug, and the difference between the dotted and broken horizontal lines was taken as the maximal possible reduction. Response to a drug was expressed as % of this value (% maximal reduction, PMR).

Table III gives the mean PMR values obtained with dequalinium, hedaquinium, and benzylpenicillin in prophylactic, simultaneous and therapeutic tests. Results with oxytetracycline, cetrimide, and procaine penicillin in simultaneous tests are also given. It will be seen from Table III that in each type of test with each drug mean PMR increased with dose.

TABLE III

REDUCTION OF LESIONS DUE TO *C. OVIS* IN GUINEA-PIG SKIN BY ANTIBACTERIAL AGENTS ADMINISTERED INTRADERMALLY 30 MIN. BEFORE, SIMULTANEOUSLY WITH, OR 30 MIN. AFTER INJECTIONS OF *C. OVIS* AT THE SAME SITE

Mean PMR refers to mean % maximal reduction, namely % of the difference between mean lesion diameters after *C. ovis* alone and after saline alone.

Drug	Dose/ Site (μg.)	Mean PMR		
		Drug before *C. ovis*	Drug with *C. ovis*	Drug after *C. ovis*
Dequalinium chloride	0·05	22·9	43·8	24·2
	0·20	35·5	53·2	30·8
	0·80	65·4	55·4	40·9
Hedaquinium chloride	0·05	23·0	48·0	24·4
	0·20	30·1	55·2	31·6
	0·80	41·4	61·8	38·9
Benzylpenicillin	100	35·0	26·6	21·0
	400	38·9	37·9	26·9
	1,600	45·8	42·2	32·5
Oxytetracycline hydrochloride	56·25	—	8·0	—
	75·00	—	14·1	—
	100·00	—	15·7	—
Cetrimide	1·25	—	13·6	—
	2·50	—	39·8	—
	5·00	—	53·4	—
Procaine penicillin	62·5	—	6·2	—
	250·0	—	19·1	—
	500·0	—	20·3	—

Cortisone.—Two guinea-pigs received 0.05, 0.2, and 0.8 mg. cortisone and 2 pigs received 0.05, 0.2, and 0.8 μg. dequalinium in each of 4 sites. In addition all animals were given 4 control injections of 0.05 ml. saline. Thirty min. later *C. ovis* was superinjected at all sites. Dequalinium produced the expected reduction in lesion diameter, but no effect was observed after cortisone.

Drugs without C. ovis

Antibacterials.—All drugs at high doses exhibited toxicity, except procaine penicillin, which was too insoluble to allow sufficient intradermal doses to be given in 0.1 ml. volumes. Toxic lesions resembled those of *C. ovis*, but were flatter, and their diameters increased with increasing dose, as will be seen for benzylpenicillin and hedaquinium in the right-hand portions of Figs. 4 and 5. For reasons discussed below, we have taken as a practical measure of intradermal toxicity the maximal tolerated intradermal dose (MTID), which is the greatest dose producing a lesion no larger than that caused by the saline control injection.

Diphtheria Toxin.—When either dequalinium at 0.8 μg. or benzylpenicillin at 0.8 mg. were injected simultaneously with Schick toxin, the resulting lesions were not significantly smaller than those produced by toxin alone. The lesion produced by Schick toxin control was not significantly larger than that of saline control.

Old Tuberculin.—Six guinea-pigs, sensitized with killed *M. tuberculosis*, and treated subcutaneously with cortisone, and 6 other sensitized guinea-pigs, untreated with cortisone, were each given 6 intradermal injections of old tuberculin in doses of 160, 40, or 10 units. Analysis of variance showed that cortisone gave a highly significant reduction ($P<0.001$) in weal diameter, in confirmation of the finding of Long and Miles (1950).

In another type of experiment 9 guinea-pigs sensitized to *M. tuberculosis* were used. All animals received saline intradermally at two sites, of which three received 0.2 and 0.8 mg. cortisone, three 0.2 and 0.8 μg. dequalinium and three 0.2 and 0.8 μg. hedaquinium intradermally at each of two sites. All sites were superinjected with 100 units of old tuberculin. None of the intradermal treatments significantly reduced lesion diameter compared with the control mean ($P>0.05$).

DISCUSSION

Validity of Method

The question arose as to whether the antibacterial substances studied reduced the lesion of *C. ovis* by directly neutralizing bacterial toxin, by anti-inflammatory action or by damaging the bacteria.

The failure of benzylpenicillin and dequalinium to reduce the specific reaction to diphtheria toxin shows that they almost certainly did not act as antitoxic agents. We regard this result as typical of all antibacterial agents used.

Systemic cortisone reduced the response to old tuberculin; but intradermal dequalinium and hedaquinium did not. Cortisone, given intradermally, failed to reduce either the old tuberculin or *C. ovis* lesions. These facts made it highly improbable that the antibacterial compounds,

which were always given intradermally, acted as anti-inflammatory agents.

Since antitoxic and anti-inflammatory actions have been excluded, there remains the hypothesis that the antibacterials reduced the *C. ovis* lesions by direct damage to the bacteria. Two pieces of evidence are consistent with this hypothesis. First, all active substances were found to inhibit *C. ovis* strongly *in vitro*. Second, killed bacteria did not produce the typical lesion and bacteria damaged by penicillin before inoculation only produced a lesion at a time after injection which corresponded to the reappearance of growth *in vitro*.

Measurement of Toxicity

We have considered toxicity only as an intradermal response, since this tends to oppose any intradermal therapeutic effect. The curve relating log dose to diameter of toxic lesion might be expected on general grounds to be sigmoid; but the lower portion of this curve cannot be followed below the point at which saline itself produces a lesion, and we did not increase drug dosage to the point where the upper portion of the curve presumably becomes asymptotic (Figs. 4 and 5). These conditions made it impossible to measure toxicity at the point half-way between the upper and lower asymptotes which corresponds to the LD50 in dose/lethality curves. However, the truncation of the lower portion of the intradermal dose/response curve made it possible to express toxicity with reasonable precision as the maximal tolerated intradermal dose (MTID), which corresponds to the point at which the dose/response curve for toxicity meets or extrapolates to the horizontal of the saline lesion.

Measurement of Antibacterial Efficiency in vivo

To measure the efficiency of a drug *in vivo*, it is usual to relate therapeutic potency to toxicity. In experimental infections of the whole animal, this is done by means of the therapeutic ratio. Since, in the present work, such a measure did not apply, we used such graphs as those in Figs. 4 and 5 to relate intradermal antibacterial action to intradermal toxicity.

For two reasons, lesion diameter reached a minimum in each type of test at an optimal dose which was the MTID. First, none of the drugs was 100% effective at doses lower than the MTID, and at higher doses toxic effects opposed antibacterial effects. Second, the slope of the toxicity curve for each antibacterial drug studied was considerably greater than that for the antibacterial activity.

In Figs. 4 and 5, the lesion diameter curves have been extrapolated to cut, at P, S, and T, an ordinate erected at the MTID. The minimal lesions are indicated by these points, from which maximal PMR values have been derived. These and the corresponding MTID values are summarized in Table IV for all antibacterials used, except procaine penicillin.

TABLE IV

THE INTRADERMAL TOXICITIES OF SOME ANTIBACTERIAL AGENTS AND THEIR OPTIMAL EFFICIENCIES AGAINST EXPERIMENTAL *C. OVIS* LESIONS IN GUINEA-PIG SKIN

MTID refers to the maximal tolerated intradermal dose; for explanation of PMR, see the legend to Table III.

Drug	MTID (μg./Site)	PMR at MTID		
		Drug 30 min. before *C. ovis*	Drug Simultaneously with *C. ovis*	Drug 30 min. after *C. ovis*
Dequalinium chloride	1·6	67·8	60·2	44·1
Hedaquinium chloride	1·0	43·1	62·9	39·6
Benzylpenicillin ..	2,500	48·0	45·0	34·0
Oxytetracycline hydrochloride ..	79·4	—	15·8	—
Cetrimide	4·2	—	54·2	—

If drugs capable of reducing lesion diameter of *C. ovis* to that of the saline controls are to be compared, their relative merits might be measured as the ratio of the MTID to the smallest dose which produces 100% reduction of lesion diameter (minimal effective dose). This measure would correspond to a conventional therapeutic ratio.

Relation to Clinical Findings

It will be seen from Table III that almost all values obtained with dequalinium and hedaquinium were comparable. From Table IV it appears that benzylpenicillin possessed about three-quarters of the efficiency of dequalinium. Cetrimide was intermediate between these two drugs whereas oxytetracycline was only slightly active.

Several factors make it difficult to relate these results to those of clinical practice. First, many species of bacteria other than *C. ovis* are involved; second, natural lesions are usually well established before they are treated topically; and third, treatment usually consists in applying creams or paints. Nevertheless, our results with *C. ovis* probably agree with clinical impressions, in that all antibacterials used were somewhat, but not completely, effective.

We feel that the method described for evaluating topical antibacterials could be further developed, using other organisms more frequently met in

clinical practice. It seems reasonable, however, to suppose that if a drug was effective in this test against *C. ovis* and was active *in vitro* against another species, it would also act topically against the latter species.

We wish to thank Professor A. A. Miles for his advice and for the strain of *C. ovis*, Dr. A. Patterson for the preparation of *M. tuberculosis* and Mr. H. J. Fraser and Mr. G. K. A. Smith for photography. This work was carried out with the technical assistance of Miss Margery Boothroyd. Some preliminary experiments on other species were carried out by Mrs. M. Babbs.

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THE SCHISTOSOMICIDAL AND TOXIC EFFECTS OF SOME αω-DI(*p*-AMINOPHENOXY)ALKANES AND RELATED MONOAMINES

BY

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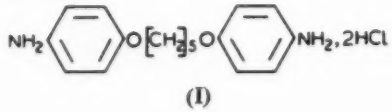
An account is given of the results obtained with a number of *p*-aminophenoxyalkanes which were examined for activity against *Schistosoma mansoni* in mice and for the presence of retinotoxicity in cats. The first compound to be found active, 1 : 5-di(*p*-aminophenoxy)-pentane dihydrochloride (M & B 968A), was modified in a variety of ways in an attempt to increase its activity and to eliminate its ocular effects. It was found that a *p*-aminophenoxyalkyl group was common to all the active compounds, but that the rest of the molecule could be any of several different groups, including alkoxy, phenoxy, and phenyl groups, without diminishing the activity. Although at least one *p*-aminophenoxyalkyl group was essential for schistosomicidal activity, ocular toxicity was also shown by a *p*-aminophenylalkyl derivative in which the ether linkage was absent.

The standard remedies for schistosomiasis are tartar emetic, stibophen, antimony lithium thiomalate (Anthiomaline) and lucanthone (Miracil D), a thioxanthone derivative first prepared by Mauss (1948). None of these compounds is completely satisfactory because the antimonials must be given at near toxic doses in order to cure the disease, and lucanthone, in addition to its unpleasant side-effects, is inactive against the oriental species *Schistosoma japonicum*.

Over the past few years a large number of compounds has been screened in these laboratories for activity against *S. mansoni* in mice. During the early phases of this investigation some benzthiazole derivatives were prepared by our colleague Mr. S. S. Berg. These proved to be of no biological interest, but an intermediate from this work, 1 : 5-di(*p*-aminophenoxy)pentane dihydrochloride (M & B 968A) (I), was found to be highly active. No compound of this type had previously been shown to possess marked schistosomicidal activity although a related compound, 1 : 5-di(*p*-amidinophenoxy)pentane diisethionate (pentamidine), was found by Boiron and Koerber (1947) to possess slight though transient activity against *S. haematobium* in man.



(I)

This discovery was made in 1952 and led to the initiation of an extensive chemical programme which is still going on (Collins, Davis and Hill, 1954). In the same year, activity was found quite independently by Raison and Standen (1955) in the very closely related compound, 1 : 5-di(*p*-methylaminophenoxy)pentane dihydrochloride. Consequently two groups of workers have quite independently set out from virtually the same chemical starting point and followed paths which have sometimes crossed but more often diverged. Inevitably the two research studies overlap to some extent, especially in the early phases (Raison and Standen, 1955; Caldwell and Standen, 1956; Standen and Walls, 1956; Gorvin, Raison, Solomon, Standen and Walls, 1957).

The present paper therefore deals mainly with compounds similar to those already described by Standen and his colleagues, but it also includes the results of certain toxicological studies in cats to which we attach considerable importance. Some of the compounds studied have been similarly examined by Goodwin, Richards, and Udall (1957). Our therapeutic results with these compounds agree very well with those of Standen, although there are minor differences which are discussed below.

M & B 968A was found by Hill (1956) to be as active as lucanthone against *S. mansoni* in mice and much less toxic, but in monkeys it was almost

inactive and produced retinotoxic effects, which have also been observed in cats and dogs, but not in other species (Edge, Mason, Wien, and Ashton, 1956). The histology of the eyes has been studied only in cats (Ashton, 1957), the changes resembling those produced by sodium iodate. Destruction of the visual cells, localized displacement of the outer nuclear layer, and marked degeneration and proliferation of the pigment epithelium occurred; the bipolar and ganglion cells were unaffected.

It was therefore essential to know if active compounds produced effects on the eye before attempting a clinical trial in man. Consequently many of them were examined in cats to determine whether they produced the initial weakening of the light and blink reflexes which accompany visual impairment.

METHODS

Schistosomicidal Activity in Mice.—The methods employed have been fully described by Hill (1956). Mice infected with *S. mansoni* seven to eight weeks previously were dosed by mouth daily for four days. Fifteen mice were used for each dose; one group of 15 untreated controls was used in each experiment.

Two types of therapeutic experiment were carried out: (*a*) a preliminary screening test in which a dose equivalent to about one-third of the LD50 was given each day; and (*b*) a more detailed examination of active compounds in which several doses were given to obtain a dose/response curve.

In the preliminary test, the mice were killed and examined about one week after the end of treatment and the number of living worms recovered from the mesenteric veins, portal vein, and liver was compared with the number from the untreated controls. Compounds which failed to reduce the number of worms significantly (by the "*t*" test, $P=0.01$) were classified as inactive; a 50% reduction was barely significant ($P=0.05$), whereas a 70% reduction was usually significant ($P=0.01$).

In the second type of experiment, the mice were examined two weeks after the end of treatment and the number cured was determined for each dose as described by Hill (1956), and a dose/response curve was drawn from which the CD50 and the CD95 were obtained graphically. Our methods, like those of Raison and Standen (1955), measured essentially the drift of worms back to the liver after treatment and their subsequent ensheathment and death, but, whereas Raison and Standen expressed their results as the % of worms killed, we expressed ours as the % of mice cured.

The acute oral toxicity of each compound was determined in groups of five mice. The LD50 was obtained graphically. Chronic oral toxicity experiments using 10 mice/dose were carried out with most of the active compounds. The mice were treated daily for four days, as in the therapeutic experiments, and the LD50 and the LD5 were again determined graphically. This enabled therapeutic ratios, LD50/CD50 and LD5/CD95, to be calculated for each compound, although only the LD50 and the CD50 are given here.

Toxicity in Cats.—Healthy, adult cats of both sexes were used. The compounds were administered orally in soluble capsules or sometimes, when sufficiently soluble, by subcutaneous injection. Usually each compound was administered on a single occasion to two cats at a dose of approximately 0.4 g./kg. or less if the compound proved toxic at this dose. The animals were frequently (twice daily) examined for the development of ocular effects and were destroyed immediately such effects were observed. Whereas negative results obtained with two cats are not particularly significant for individual compounds, they do make it possible to distinguish less toxic series where none of the members tested gave a response. On the other hand, a positive response in only two cats could be regarded as a definite effect. Other toxic effects such as ataxia, muscular weakness, and vomiting were seen in some of the cats.

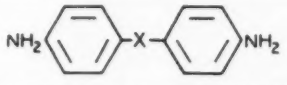
RESULTS

Schistosomicidal Effects

Modification of the Central Chain.—Various modifications of the central chain of M & B 968A are shown in Tables I and II. The methane, ethane, decane and dodecane homologues (Table I) were inactive, but compounds with three to nine carbon atoms in the chain were active, with a peak at about seven carbon atoms. These results accord well with those of Raison and Standen (1955).

TABLE I

DIAMINES, MODIFICATION IN THE NUMBER OF CARBON AND OXYGEN ATOMS IN THE CENTRAL CHAIN

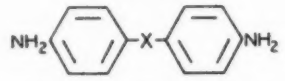


All compounds were tested as the dihydrochlorides except those marked (b) dimethanesulphonates. The following compounds were also therapeutically inactive: X = $O[CH_2]_2O[CH_2]_2O$; $O.CO[CH_2]_3CO.O$; $S[CH_2]_2S$; $O[CH_2]_3NH$; $NH[CH_2]_3NH$; $NAc[CH_2]_3NAc$; CH_2; $[CH_2]_2$; CH_2OCH_2; CH=CH; CO; NH; SO_2; SO; SS; S.SO; S; O; single bond.

M & B No.	X	LD50 g./kg. Single Dose	LD50 g./kg. Daily Dose	CD50 g./kg. (Daily Dose)	Ocular Effects
2496	OCH_2O	1·0		Inactive	+
966A	$O[CH_2]_3O$	0·75		"	+
1113A	$O[CH_2]_5O$	1·0	0·67	0·35	+
2467	$O[CH_2]_4O$	1·0	0·32	0·13	+
968A	$O[CH_2]_5O$	0·75	0·62	0·19	+
2491	$O[CH_2]_6O$	1·5	0·17	0·08	+
2539	$O[CH_2]_7O$	1·5	0·33	0·07	+
2512	$O[CH_2]_8O$	>4·0	0·42	0·08	+
2524	$O[CH_2]_9O$	2 5	0·27	0·1	+
2514	$O[CH_2]_{10}O$	>4·0		Inactive	+
2612	$O[CH_2]_{12}O$ (b)	>8·0		"	−
2952	$O[CH_2]_5$ (b)	1·5	0·75	0·2	+
2562	$[CH_2]_8$ (b)	0·8		Inactive	+

TABLE II

DIAMINES, REPLACEMENT OF THE CENTRAL CHAIN BY BRANCHED OR UNSATURATED CHAINS OR CARBOCYCLIC RINGS



All compounds were tested as the dihydrochlorides except those marked (a) bases or (b) dimethanesulphonates. The following compounds were also therapeutically inactive: X = *p*-$OCH_2C_6H_4CH_2O$; *p*-$O[CH_2]_5OC_6H_4O[CH_2]_5O$.

M & B No.	X	LD50 g./kg. Single Dose	LD50 g./kg. Daily Dose	CD50 g./kg. (Daily Dose)	Ocular Effects
2579	$OCH_2CH(OH)CH_2O$ ..	1·5	0·56	Inactive	
2576	$O[CH_2]_2CHMe[CH_2]_2O$ ..	0·6		0·33	+
2820	$O[CH_2]_2CMe_2[CH_2]_2O$ ..	0·5		Inactive	
2595	$O[CH_2]_2CHPh[CH_2]_2O$ ..	0·75		,,	
2869	1 : 5-dioxy-3 : 3-pentamethylenepentane	3·0		,,	
2734	$OCH_2C(CH_2OC_6H_4$-*p*-$NH_2)_2CH_2O$ (b)	2·0	0·65	>1·0	
2673	*trans*-OCH_2CH=$CHCH_2O$ (a)	0·75	0·5	0·13	+
2676	*cis*-OCH_2CH=$CHCH_2O$ (a)	1·5		0·22	+
2616	$OCH_2C{\equiv}CCH_2O$ (b) ..	>4·0		Inactive	
2720	$OCH_2C{\equiv}C.C{\equiv}CCH_2O$ (a)	2·0		,,	
2451	*p*-OC_6H_4O	0·7	0·3	0·24	
2610	1 : 4-dioxy*cyclo*hex-2-ene (b)	0·75	0·29	>0·29	+
2667	1 : 4-dioxy*cyclo*hexane (b)				

Omission of both ether linkages (Table I) resulted in complete loss of activity, but the omission of only one ether linkage had no effect, which demonstrates that only one *p*-aminophenoxyalkyl group is essential for activity.

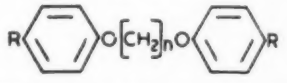
Branched chains (Table II) had a deleterious effect, as Gorvin *et al.* (1957) also found, but compounds with a double bond in the chain were as active as those with a saturated chain. It is doubtful if the apparent difference in activity between the *cis* and *trans* isomers was significant. The two compounds with a triple bond which we examined were completely inactive although Gorvin *et al.* (1957) found activity in some acetylenic compounds, the activity increasing with the distance between one oxygen atom and the triple bond. The replacement of the central chain with a carbocyclic ring was also dystherapeutic, the activity decreasing from *cyclo*hexene through *cyclo*hexane to benzene.

Substitution in the Amino Groups.—A number of secondary and tertiary amines and one quaternary ammonium compound were tested (Table III). Among the secondary amines activity was found in the methyl, ethyl, hydroxyethyl and *n*-propyl derivatives, but not in the *iso*propyl derivative, whereas among the tertiary amines only the methyl, ethyl and hydroxyethyl derivatives were active. The secondary and primary amines were equally effective but some of the tertiary amines were less so, and the quaternary ammonium compound was inactive.

This field was covered thoroughly by Raison and Standen (1955) and Gorvin *et al.* (1957). Our results agree very well with theirs with the single exception of the *iso*propyl derivative, which Raison and Standen (1955) found to be active.

TABLE III

DIAMINES, SUBSTITUTION IN THE AMINO GROUPS



All compounds were tested as the bases except those marked (c) dihydrochlorides or (d) disulphates. The following compounds were also therapeutically inactive: n = 3, R = NHAc; N:CHPh; n = 5, R = $NH[CH_2]_2NEt_2$; $NHCO_2Et$; $NHC(:S)NH_2$; $NHC(:NH)NH_2$; $NHCH_2Ph$; pyrid-2-ylamino; 4-methylthiazol-2-ylamino; pyrrolidin-1-yl; piperidino; morpholino. M & B No. 2592 was tested in one cat.

M & B No.	n	R	LD50 g./kg. Single Dose	LD50 g./kg. Daily Dose	CD50 g./kg. (Daily Dose)	Ocular Effects
2519	3	NHMe (c)	1·5	0·6	0·32	+
2526	5	NHMe (c)	3·0	0·61	0·16	+
2841	8	NHMe (c)	1·8	0·31	0·065	−
2592	3	NMe_2	4·0	0·82	0·39	−
2542	5	NMe_2 (c)	4·0	0·6	0·33	+
2668	8	NMe_2	>4·0		>1·6	−
2507	3	NMe_3I^-	0·5		Inactive	
2672	5	NHEt	3·0	0·54	0·14	+
2651	5	NEt_2	>4·0	0·55	0·24	−
2694	5	NHPr (*n*)	>4·0	0·73	0·19	+
2658	5	NHPr (*iso*)	>4·0		Inactive	
2714	5	NPr_2(*n*)(d)	>4·0		,,	−
2782	5	$NHCH_2CH_2OH$ ..	4·0	1·1	0·19	+
3346	5	$N(Me)CH_2CH_2OH$ ..	>4·0		<1·0	
2598	5	$N(CH_2CH_2OH)_2$ ..	>8·0	1·7	0·27	+
3001	8	$N(CH_2CH_2OH)_2$ ..	>4·0		<1·0	+
3106	5	$NHCH_2CH_2OMe$ ..	>4·0	0·38	0·37	+
2440	5	$NHCH_2SO_3Na$ ..	>2·0	3·2	0·41	+

Substitution in the Nucleus.—All the disubstituted compounds of this type (Table IV) were inactive except the *oo'*-dimethoxy derivative, which showed very slight activity at a toxic dose. One monosubstituted compound, 1-(4-amino-2-bromophenoxy)-3-phenoxypropane dihydrochloride, was found to be effective, although less so than the parent compound (M & B 1113A, Table I).

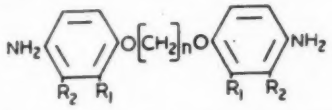
Standen and Walls (1956) also found that nuclear substitution resulted in a great reduction in activity and that the effect was greater when both rings were substituted.

Position and Number of the Amino Groups.—Modification of the position and number of the amino groups demonstrated that activity was dependent on the presence of at least one *p*-amino group since compounds with the amino groups in the *oo'*- or *mm'*-positions were inactive, as was the *op'*-isomer. Slight activity was shown by a

compound with the amino groups in the *mp'*-positions. Omission of one of the amino groups increased the toxicity to mice but had little effect on activity. Omission of both amino groups resulted in complete loss of activity.

TABLE IV

DIAMINES, SUBSTITUTION IN THE NUCLEUS



All compounds were tested as bases except those marked (b) dimethanesulphonates or (c) di- or tetra-hydrochlorides. M & B Nos. 2536 and 2623 gave positive ocular responses in cats whereas M & B No. 3927 gave none.

M & B No.	n	R_1	R_2	LD50 g./kg. (Single Dose)	CD50 g./kg. (Daily Dose)
2536	5	Cl (c)	H	3·0	Inactive
2623	5	H (b)	Cl	1·5	,,
3927	5	OMe (b)	H	1·0	>0·8
2563	5	H (c)	CH_3	0·5	Inactive
2492	3	NH_2	H	1·5	,,
2497	5	H	NH_2	1·5	,,
2638	5	H	NHAc	>4·0	,,
2647	3	H	NO_2	>4·0	,,
2446	5	CH_2NEt_2 (c)	H	0·5	,,

Monoamines with Two Phenoxy Groups.—It was at first thought probable that two *p*-aminophenoxy groups were necessary for schistosomicidal activity. However, with the discovery of slight activity, at a toxic dose, in 1-*p*-aminophenoxy-3-phenoxypropane methanesulphonate, it became clear that only one such group was required and hence a new range of compounds was examined. The pentane derivative (M & B 2666, Table V) was tested next and found to be more active than the propane homologue. The effect of various substituents in the phenoxy group of M & B 2666 was then investigated, but unfortunately all of these except a *p*-acetamido substituent reduced the activity (Table V). This latter compound, which was rather more active and only about half as toxic as the parent, was later shown by our colleague Dr. Fraser to be a metabolite of M & B 968A (Table I).

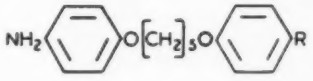
Caldwell and Standen (1956) also studied compounds of this type with substantially similar results, except for the *p*-methyl, *p*-chloro and *p*-carboxy derivatives, which they found to be very slightly active at total doses higher than those we were able to administer owing to the toxicity of the compounds.

Other Monoamines with Two Ether Linkages.—Extending the search for active monoamines, we prepared and tested some analogous alkoxy derivatives (Table VI). Apart from the benzyloxy compound (M & B 3113), however, the order of activity was low throughout this series. Caldwell and Standen (1956) also studied similar compounds and concluded that the activity was only moderate.

Monoamines with One Ether Linkage.—Of several compounds of this type which were tested (Table VII) only the 5-phenylpentyl derivative

TABLE V

MONOAMINES WITH TWO PHENOXY GROUPS

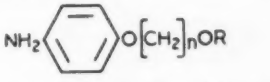


All compounds were tested as bases except those marked (b) methanesulphonates or (c) hydrochlorides. The following compounds were also therapeutically inactive: 1-*p*-aminophenoxy-5-(3:5-xylyloxy)-pentane methanesulphonate; 1-*p*-aminophenoxy-5-*o*-methoxyphenoxypentane; 1-*p*-aminophenoxy-4-phenoxy*cyclo*hexane.

M & B No.	R	LD50 g./kg. Single Dose	LD50 g./kg. Daily Dose	CD50 g./kg. (Daily Dose)	Ocular Effects
2666	H	0·75	0·49	0·21	+
2709	Cl (c)	0·5		Inactive	
2718	Me (c)	1·8		,,	
2735	CH_2NH_2 (b)	0·8		,,	
2730	OH (b)	2·0		,,	
2660	OMe	1·5	0·27	>0·27	+
2967	OEt	3·0		1·0	–
2783	OPh	1·0		Inactive	
2956	Ac	4·0		>1·0	+
2877	CO_2H (c)	0·9		Inactive	
2847	CO_2Et	>4·0		,,	
2818	NO_2	>4·0		>1·0	–
2693	NHAc	>4·0	1·1	0·15	+
3152	$NHCOCH_2OH$	0·5		Inactive	
2997	NHCOEt	>4·0		<1·0	+
3003	NHCOPh	>4·0		1·0	
3041	$NHSO_2Me$	>4·0		<1·0	–
2805	Ph (b)	>4·0		Inactive	

TABLE VI

OTHER MONOAMINES WITH TWO ETHER LINKS

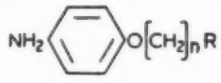


All compounds were tested as methanesulphonates except those marked (a) bases or (c) hydrochlorides.

M & B No.	n	R	LD50 g./kg. Single Dose	LD50 g./kg. Daily Dose	CD50 g./kg. (Daily Dose)	Ocular Effects
2742	3	Me	1·5		>0·4	
2836	5	Me	1·5		>0·5	–
3037	5	Et	0·5		Inactive	+
3048	5	Pr(*n*)	1·5	0·6	0·35	+
3113	5	CH_2Ph	0·75	0·64	0·095	+
3057	5	CH_2CH_2OH (a)	2·0	0·94	0·77	–
3066	5	CH_2CH_2OMe	0·7		Inactive	
3675	5	2-phthalimidoethyl	>4·0		0·6	
2775	5	naphth-1-yl	1·6		Inactive	
2844	5	naphth-2-yl (a)	4·0		,,	
3306	5	pyrid-2-yl (a)	3·0		>0·5	
2770	5	quinol-6-yl (c)	0·6		Inactive	
3026	5	*cyclo*hexyl	0·25		,,	

(M & B 2719) was active. However, this result in conjunction with that found for M & B 2952 (Table I) shows that only one ether linkage is essential for activity. The inactivity of the propane homologue was possibly due to its high toxicity which meant that only a very small dose could be given in the therapeutic experiment. All the compounds of this type which Caldwell and Standen (1956) examined were inactive.

TABLE VII
MONOAMINES WITH ONE ETHER LINK



All compounds were tested as bases except those marked (b) methanesulphonates or (c) hydrochlorides.

M & B No.	n	R	LD50 g./kg. Single Dose	LD50 g./kg. Daily Dose	CD50 g./kg. (Daily Dose)	Ocular Effects
2652	0	H	0·75		Inactive	–
2417	1	H (c)	1·5		,,	–
2415	2	H (c)	1·0		,,	–
2839	6	H (b)	0·07		,,	+
2655	10	H	1·5		,,	–
2843	0	*cyclo*hex-2-en-1-yl	0·03		,,	
2817	0	*cyclo*hexyl (b)	0·02		,,	
3837	3	$CH=CH_2$ (b)	0·15		,,	
3933	3	$CH(OEt)_2$	1·5		,,	
2701	3	OH (b)	>4·0		,,	
2729	5	OH	2 0		,,	
2685	3	OAc	1·5		,,	
2754	1	CO_2H	>4·0		,,	
2966	4	CO_2H	1·5		,,	
2857	5	CO_2H	>4·0		,,	
2840	1	$CONH_2$	>4·0		,,	
3039	5	$CONH_2$	>4·0		,,	
3017	0	Ph (c)	0·12		,,	
2926	2	Ph (b)	0·2		,,	
2883	3	Ph	0·08		,,	
2719	5	Ph (b)	1·5	0·37	0·08	+

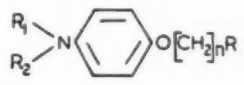
Monoamines, Substitution in the Amino Group. Only a few of these compounds were examined (Table VIII) and all but two of them were active.

TABLE VIII
MONOAMINES, SUBSTITUTION IN THE AMINO GROUP

All compounds were tested as bases except the one marked (c) hydrochloride. M & B No. 3011 gave a positive ocular response in cats.

M & B No.	n	R	R^1	LD50 g./kg. (Single Dose)	CD50 g./kg. (Daily Dose)
3568	8	NHMe	OPh	3·0	>0·8
3383	5	NMe_2	*p*-OC_6H_4NEtAc	1·8	0·4
3506	5	NHEt (c)	Ph	1·5	<0·4
3112	5	$N(CH_2CH_2OH)_2$	Ph	3·0	<1·0
3011	5	$N(CH_2CH_2OH)_2$	*p*-OC_6H_4NHAc	>4·0	<1·0
2763	5	$NHCH_2CH_2NEt_2$	OPh	0·8	Inactive
2785	5	$N(CH_2CH_2NEt_2)_2$	OPh	0·5	,,

Molecular Features Essential for Schistosomicidal Activity.—



(II)

A very wide range of compounds with the above general formula (II) (where n=3 to 9; R_1 and R_2=H, lower alkyl or hydroxyalkyl; and R= alkoxy, phenoxy, or phenyl, which may be substituted) was found to be active. The *p*-aminophenoxypropyl moiety common to all of them appeared to be essential for activity, since compounds without a *p*-amino group, without an ether linkage, or with less than three carbon atoms in the chain were inactive. This conclusion differs from that of Caldwell and Standen (1956), who considered that two ether linkages were necessary for activity. Our results have demonstrated that only the ether linkage present in the *p*-aminophenoxyalkyl group is essential.

Ocular Effects

Diamines.—All the compounds in Tables I and II except the dodecane compound (Table I) which were examined for ocular effects in cats gave a positive response.

Ten substituted amines (Table III) were retinotoxic, but five others, four of them tertiary amines, failed to produce an effect.

Among the nuclear substituted diamines (Table IV) the *oo'*- and *mm'*-dichloro derivatives both produced ocular symptoms. The *oo*-dimethoxy derivative gave no ocular response, but it produced ataxia and muscular weakness in cats, symptoms which sometimes accompanied the disappearance of ocular effects in other compounds.

Among the tertiary amines we found the hydroxyethyl derivatives of the pentane and octane member to be retinotoxic, although Goodwin *et al.* (1957) found that the corresponding heptane did not possess this property.

Variation in the Position and Number of the Amino Groups in M & B 968A.—Two compounds with amino groups in the *oo*- or *mm*-positions produced no retinal effects in cats, but those with amino groups in the *mp'*-, *op'*- or *p*-positions were retinotoxic.

Monoamines.—Among the monoamines (Tables V, VI, VII, and VIII) the 5-*p*-acetamidophenoxy, 5-benzyloxy and 5-phenyl derivatives showed sufficient schistosomicidal activity to be of interest, but they all produced ocular symptoms in cats, whereas

the compounds which gave no response in cats had small therapeutic ratios or were inactive against *S. mansoni*. 1-p-Aminophenoxy - 5 - methoxypentane and the corresponding 5-ethoxy derivative (Table VI) both produced ataxia and muscular weakness in cats. 1-*p* - Aminophenoxyhexane (Table VII) also produced ataxia and muscular weakness in addition to its effects on the eye. The toxic symptoms due to *p*-aminophenol, *p*-anisidine and *p*-phenetidine (Table VII) consisted in cyanosis of the mouth and tongue, rapid shallow respiration, and oedema of the face and paws.

Molecular Features Essential for the Production of Retinotoxicity.—The molecular features essential for the production of retinotoxicity were not identical with those required to produce schistosomicidal effects since M & B 2562 (Table I), which had no ether linkage and was consequently not schistosomicidal, was nevertheless toxic to the eye. Also M & B 3927 (Table IV), which showed slight schistosomicidal activity, was non-retinotoxic although its unsubstituted parent compound (M & B 968A, Table I) was highly retinotoxic. This suggests that nuclear methoxy substituents may inhibit the production of ocular effects, although chloro substituents do not (Table IV).

As far as can be judged from the present results a *p*-amino group is necessary for both schistosomicidal activity and ocular toxicity.

We wish to thank our colleagues, Drs. J. N. Ashley, H. J. Barber and R. Wien, for their helpful discussions during this investigation, Miss N. E. Sirett for the preparation of some of the compounds, Mr. D. F. J. Mason for carrying out some of the experiments in cats, and Mr. A. D. Speed for his technical assistance.

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THE TRYPANOCIDAL ACTIVITY OF SOME PYRIMIDYLAMINOPHENYLARSONIC COMPOUNDS

BY

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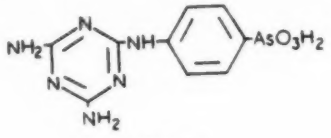
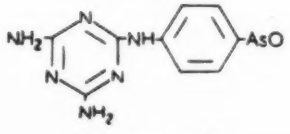
(RECEIVED MARCH 1, 1958)

A series of substituted pyrimidylaminophenylarsonic acids have been prepared, reduced to the corresponding oxophenylarsines and finally, in certain cases, to the arsenobenzenes. The tervalent arsenicals were highly active against *Trypanosoma rhodesiense* in mice. 4-(2:6-Diaminopyrimid-4-ylamino)phenyloxoarsine either as the insoluble carbonate or the soluble isethionate was the most promising of these compounds.

The announcement by Friedheim in 1939 of the new arsenical drug, melarsen, for the treatment of human trypanosomiasis followed by the introduction in 1944 of the more highly active derivative, melarsen oxide, stimulated work in the field of arsenical compounds containing heterocyclic nuclei. Although most of the early work had been carried out against *Trypanosoma equiperdum* in mice, Friedheim stated in 1941 that he had obtained favourable results in the treatment of man infected with *T. gambiense*.



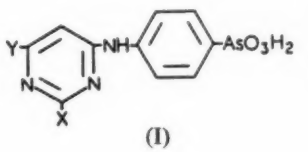
Melarsen



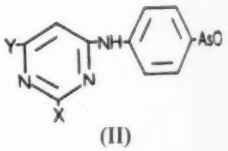
Melarsen oxide

Following the publication of the account of melarsen oxide, Banks and Controulis (1946) described analogous pyrimidylaminophenylarsonic acids and some derivatives of these compounds containing tervalent arsenic. However, the trypanocidal activity of these compounds against *T. equiperdum* in rats was inferior to that of the previously prepared arsenicals of the anilinotriazine series.

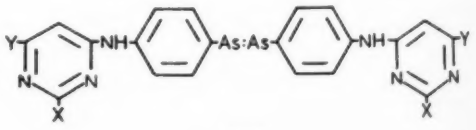
Our interest lay in determining the activity of arsenical compounds containing a pyrimidine nucleus against *T. rhodesiense* and so assessing their possible value for the treatment of human trypanosomiasis. Accordingly a series of pyrimidylaminophenylarsonic acids were prepared having various substituents in the 2- and 4-positions of the pyrimidine nucleus (I) as well as compounds containing a pyrimidinium radical. Some of these compounds were reduced (*a*) by treatment with sulphur dioxide and hydriodic acid to the corresponding oxophenylarsines (II) and (*b*) by hypophosphorous acid to the *pp'*-di(pyrimidylamino)arsenobenzenes (III). All the compounds were then tested biologically.



(I)



(II)



(III)

METHODS AND MATERIALS

Chemical

The compounds described in Tables I to V were prepared by the general procedures given for these types of substances. Analytical details are given in Table VI. In the cases where the preparative method differed appreciably from the general method the preparations are described in detail.

Pyrimidylaminophenylarsonic Acids (I)

Preparation.—The substituted pyrimidine (0.1 mole), arsanilic acid (0.1 mole), water (120 ml.) and concentrated hydrochloric acid (10 ml.) were boiled under reflux for 1.5 hr. After cooling the solid was collected and crystallized from water containing some hydrochloric acid (acid strength approximately 0.1N) to give the hydrochloride of the required pyrimidylaminophenylarsonic acid.

2-Amino-4-(p-arsonophenylamino)-1:6-dimethylpyrimidinium Chloride. — 2-Amino-4-chloro-1:6-dimethylpyrimidinium iodide (5.72 g.) was dissolved in hot water (50 ml.) and shaken vigorously for 3 min. with freshly prepared silver chloride (14.3 g.). The hot suspension was filtered, the filtrate added to the hot solution of arsanilic acid (4.34 g.) in a mixture of water (28 ml.) and concentrated hydrochloric acid (2 ml.) and this mixture boiled under reflux for 15 min. Excess sodium chloride was added and the whole cooled in ice when colourless prisms separated. These were collected and crystallized from 0.2N aqueous hydrochloric acid when colourless needles of 2-amino-4-(*p*-arsonophenylamino)-1:6-dimethylpyrimidinium chloride monohydrate were obtained, m.p. 336 to 337° with decomposition. (Found: C, 36.5; H, 4.9; N, 13.9; As, 19.1. $C_{12}H_{16}O_3N_4AsCl,H_2O$ requires: C, 36.7; H, 4.6; N, 14.3; As, 19.1%.) This substance lost hydrogen chloride on crystallization from water, particularly if the *p*H of the solution was adjusted to 5 by the addition of dilute sodium carbonate solution, to give the anhydro-base in the form of heavy, small prisms which were insoluble in water and had m p. 344° (decomp.). The quaternary chloride was readily reformed on treatment with dilute hydrochloric acid.

2:6-Diamino-4-(p-arsonophenylamino)-1-methylpyrimidinium Chloride. — 2:6-Diamino-4-chloro-1-methylpyrimidinium iodide (5.7 g.) was dissolved in boiling water (120 ml.) and the hot solution shaken with excess freshly precipitated silver chloride. The mixture was filtered and to the filtrate arsanilic acid (8.7 g.) was added and the whole boiled under reflux for 5 hr. Fine colourless needles separated on cooling; these were collected and repeatedly crystallized from 1% aqueous hydrochloric acid when 2:6-diamino-4-(*p*-arsonophenylamino)-1-methylpyrimidinium chloride monohydrate was obtained, m.p. 265° (decomp.). (Found: C, 33.6; H, 4.8; N, 17.9; As, 19.1. $C_{11}H_{15}O_3N_5AsCl,H_2O$ requires: C, 33.6; H, 4.2; N, 17.8; As, 19.1%.)

2:4-Diamino-6-(p-arsonophenylamino)-1-methylpyrimidinium Chloride. — 2:4-Diamino-6-chloro-1-methylpyrimidinium iodide (5.7 g.) was converted to the corresponding chloride as above, the aqueous solution added to the solution of arsanilic acid (8.7 g.) in 4% sodium hydroxide solution (40 ml.) and the whole boiled under reflux for 5 hr. The solution was then treated with decolorizing carbon, filtered, acidified to congo red paper with hydrochloric acid, excess sodium chloride added and finally cooled in ice. The crystalline material which separated on cooling was collected and the filtrate allowed to stand for 2 days. The solid which slowly separated was filtered off, extracted with 10% aqueous sodium carbonate solution, filtered from a trace of insoluble matter, acidified with acetic acid and the solution saturated with sodium chloride. The solid which slowly crystallized was filtered and repeatedly crystallized from 1% aqueous hydrochloric acid to yield 2:4-diamino-6-(*p*-arsonophenylamino)-1-methylpyrimidinium chloride monohydrate, m.p. 289° (decomp.). (Found: C, 33.6; H, 4.3; N, 17.2; As, 18.8. $C_{11}H_{15}O_3N_5AsCl,H_2O$ requires: C, 33.6; H, 4.3; N, 17.8; As, 19.1%.)

2-(p-Arsonophenylamino)-4:6-dimethylpyrimidine. — 2-Chloro-4:6-dimethylpyrimidine (2.85 g.) was added to a solution of arsanilic acid (4.34 g.) in water (100 ml.) containing 8% aqueous hydrochloric acid (10 ml.) and the mixture boiled under reflux for 10 min. Sufficient sodium hydroxide solution was added to dissolve the crystalline material which separated on cooling, the solution was filtered and the filtrate acidified with acetic acid. The white solid was collected and crystallized from 8% aqueous hydrochloric acid when 2-(*p*-arsonophenylamino)-4:6-dimethylpyrimidine hydrochloride was obtained as colourless needles, m.p. 246° (decomp.). (Found: C, 40.1; H, 4.1; N, 11.7; As, 20.9. $C_{12}H_{14}O_3N_3As,HCl$ requires: C, 40.1; H, 4.2; N, 11.7; As, 20.9%.)

2-Amino-5-(4-arsono-2-nitrophenylamino)-4:6-dimethylpyrimidine. — 4-Chloro-3-nitrophenylarsonic acid (35.2 g.) was suspended in water (250 ml.), sufficient sodium hydroxide added until the solution was neutral to litmus, 2:5-diamino-4:6-dimethylpyrimidine (17.5 g.) added and the whole boiled under reflux for 3 hr. The solution was made just acid to litmus with hydrochloric acid and cooled. The crude product was collected (50 g.) and crystallized from water when 2-amino-5-(4-arsono-2-nitrophenylamino)-4:6-dimethylpyrimidine dihydrate was obtained as buff-orange needles, m.p. 180° (decomp.). (Found: C, 34.3; H, 3.6; N, 16.6; As, 18.2. $C_{12}H_{14}O_5N_5As,2H_2O$ requires: C, 34.4; H, 4.2; N, 16.7; As, 17.8%.)

Oxo(pyrimidylaminophenyl)arsines (II)

General Preparation.—The pyrimidylaminophenylarsonic acid was dissolved in 12 volumes of 20% hydrochloric acid, potassium iodide (0.5 g.) added, the mixture warmed to 60° and sulphur dioxide passed in until no iodine was liberated on standing for 30 min. with the stream of sulphur dioxide stopped. The solid which separated was collected, dissolved in a slight excess of sodium hydroxide solution, filtered and the filtrate neutralized by the addition of dilute hydrochloric acid.

In the cases of the 2:4-diamino- or methylaminopyrimidine derivatives the above conditions were unsatisfactory, the modified conditions described below for the preparation of (*p*-2:4-diaminopyrimid-6-ylaminophenyl)oxoarsine being typical of the method for those derivatives.

(p-2 : 4-Diaminopyrimid-6-ylaminophenyl)oxoarsine.—*p* - (2 : 4 - Diaminopyrimid - 6 - ylamino)phenylarsonic acid (14.4 g.) was dissolved in concentrated hydrochloric acid (500 ml.) and potassium iodide (0.5 g.) dissolved in water (5 ml.) added. The stirred solution was warmed to 40° and sulphur dioxide passed through the mixture at this temperature for 2 hr. The mixture was then allowed to stand, the supernatant liquor decanted and the residue suspended in water (200 ml.). The mixture was made alkaline to brilliant yellow by the addition of 10% aqueous sodium carbonate solution (50 ml.), filtered and the solid dissolved in 2% aqueous sodium hydroxide solution (250 ml.). Carbon dioxide was then passed through this solution until it was no longer alkaline to Clayton yellow indicator, the precipitated solid collected, well washed with water, and dried.

(p-2 : 4-Diaminopyrimid-6-ylaminophenyl)oxoarsine Isethionate.—(*p* - 2 : 4 - Diaminopyrimid - 6 - ylaminophenyl)oxoarsine carbonate (5 g.) was suspended in water (50 ml.) and isethionic acid added until the resulting solution was neutral to litmus. After filtration from a trace of insoluble material the filtrate was evaporated to dryness *in vacuo*, the residue dissolved in ethyl alcohol, and the solution diluted slowly with ether when (*p*-2 : 4-diaminopyrimid-6-ylaminophenyl)oxoarsine isethionate separated as insoluble prism aggregates, m.p. 108° (eff.) after shrinking at 74 to 75°.

2 - Amino-4-(p-arsenosophenylamino)-1 : 6-dimethylpyrimidinium Iodide.—(*p*-2-Amino-6-methylpyrimid-4-ylaminophenyl)oxoarsine (8.7 g.) was suspended in β-ethoxyethanol (100 ml.), methyl iodide (4.0 ml.) added and the whole heated under reflux in the steam bath for 5 hr. After cooling the mixture was ground with acetone, filtered, and crystallized from water when 2 - amino - 4 - (*p*-arsenosophenylamino)-1 : 6-dimethylpyrimidinium iodide hemihydrate was obtained as colourless prisms, m.p. 248 to 250° (decomp.). (Found: N, 12.9; As, 16.9. $C_{12}H_{14}ON_4AsI,\frac{1}{2}H_2O$ requires: N, 12.7; As, 17.0%.)

pp′-*Di(pyrimidylamino)arsenobenzene Derivatives (III)*

General Preparation.—A solution of hypophosphorous acid (prepared from sodium hypophosphite (15 g.), concentrated hydrochloric acid (30.0 ml.), methyl alcohol (200 ml.) and hydriodic acid (0.5 ml.)) was added slowly during 25 min. to a suspension of the requisite pyrimidylaminophenylarsonic acid (0.03 mole) in 3.5% aqueous hydrochloric acid (250 ml.) previously heated to 70 to 75°, the whole operation being carried out in an atmosphere of nitrogen. The mixture rapidly turned yellow and an orange precipitate was formed. After heating for 1 hr., the mixture was cooled, filtered, and the residual solid well washed with absolute ethyl alcohol and finally with ether. The product so obtained is the required dihydrochloride.

The base was prepared by suspending the hydrochloride in water, adding sodium hydroxide solution until the mixture was just alkaline to Clayton yellow, filtering and washing thoroughly with water.

pp′ - *Di(2 - amine - 1 : 6 - dimethyl - 4 - pyrimidinium - amino)arsenobenzene Diphosphate.*—This compound was prepared similarly from 2-amino-4-(*p*-arsenosophenylamino)-1 : 6-dimethylpyrimidinium chloride and was obtained as a reddish-orange powder, m.p. 249 to 250° (decomp.). (Found: C, 36.1; H, 4.6; N, 13.8; As, 18.7. $C_{24}H_{32}O_8N_8P_2As_2,1\frac{1}{2}H_2O$ requires: C, 36.0; H, 4.4; N, 14.0; As, 18.8%.)

TABLE I

ACTIVITY AGAINST *T. RHODESIENSE* OF PYRIMIDYLAMINOPHENYLARSONIC ACIDS OF THE GENERAL FORMULA (I)

X and Y, see formula I. P = poisoned. C = cure (no trypanosomes observed in the blood during an observation period of 35 days). R = relapse (blood temporarily cleared of trypanosomes). VSL = very slight retarding effect on the course of the infection. NA = no action.

Comp. No.	X	Y	Salt	Results after a Single Subcutaneous Dose (Doses in mg./20 g.)			
				10	5	2·5	1
10,216	NH_2	CH_3	$HCl,\frac{1}{2}H_2O$	NA			
10,217	NH_2	H	HCl,H_2O	12C 2R	1C 3R	4R	
10,263	NH_2	Cl		P	NA		
10,362	NH_2	NH_2	HCl,H_2O	4C	7C 1R	2C 2R	
14,373	$NHCH_3$	$NHCH_3$	HCl			P	VSL
14,375	NH_2	$NHCH_3$	HCl		4C		1C 3R
14,376	H	$NHCH_3$	HCl,H_2O	6R			

TABLE II

ACTIVITY AGAINST *T. RHODESIENSE* OF OXO(PYRIMIDYLAMINOPHENYL)ARSINES OF THE GENERAL FORMULA (II)

X and Y, see formula II. * Doses of base. C = cure. R = relapse. VSL = very slight retarding effect on the infection.

Comp. No.	X	Y	Salt, Derivative	Results after a Single Subcutaneous Dose (Doses in mg./20 g.)						
				0·5	0·25	0·1	0·05	0·025	0·01	0·005
10,185	NH_2	H	$2HCl,\frac{1}{2}H_2O$	6C	4C	9C 1R	12C 3R	4C 6R	8R	
10,218	NH_2	CH_3				9C 2R	6C 5R	2C 8R	6R	
11,478	NH_2	CH_3	Dithioglycollate, H_2O			15C 1R	5C 5R	9R	10R	
11,523	NH_2	CH_3	1:2-Dimercaptopropanol, HCl		Toxic	3C 3R	4C 1R			
12,065/1	NH_2	NH_2	Carbonate, $2H_2O$				12C	4C 1R		
12,065/2*	NH_2	NH_2	Isethionate, $2H_2O$				35C	45C 2R	24C 5R	9C 6R
11,524	NH_2	Cl	1:2-Dimercaptopropanol	3P 1R	6VSL					
14,374	CH_3NH	CH_3NH	$\frac{1}{2}H_2O$		8C 4R	1C 8R				
14,377	NH_2	CH_3NH	H_2O			4C	9C	7C 1R	4R	
14,378	H	CH_3NH	$2H_2O$		5C 1R	4C 4R	4R			
16,523	CH_3NH	NH_2	H_2O		6C	12C	15C 1R	3C 2R		

TABLE III

ACTIVITY AGAINST *T. RHODESIENSE* OF DI(PYRIMIDYLAMINO)ARSENOBENZENES OF THE GENERAL FORMULA (III)

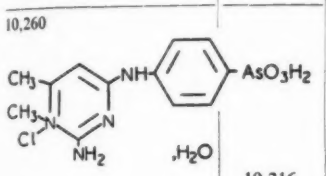
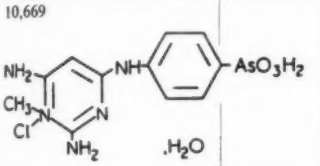
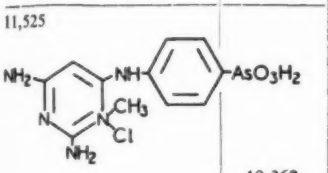
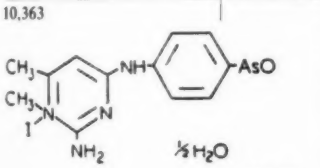
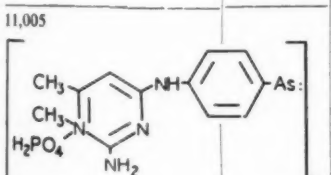
X and Y, see formula III. C = cure. R = relapse. VSL = slight retarding effect on the course of the infection. NA = no action.

Compound No.	X	Y	Salt	Results after a Single Subcutaneous Dose (Doses in mg./20 g.)				
				0·25	0·1	0·05	0·025	0·01
10,670	NH_2	CH_3	$2HCl,3\frac{1}{2}H_2O$	4C	14C 13R	5C 23R	6R	
11,444	NH_2	H	$2HCl,3\frac{1}{2}H_2O$	4C	15C 1R	18C 6R	5C 15R	1C 7R
11,476	NH_2	Cl	$1\frac{1}{2}H_2O$	VSL	NA			
11,477	NH_2	NH_2	$1\frac{1}{2}H_2O$		20C	16C 1R	13C 4R	5C 7R

TABLE IV

ACTIVITY AGAINST *T. RHODESIENSE* OF QUATERNIZED ARSENICALS

See Table I for explanation of letters in results columns.

No. and Formula	Related to	Results after a Single Subcutaneous Dose (Doses in mg./20 g.)				
		10	5	0·5	0·25	0·1
10,260 	10,216 (Table I)		NA			
10,669 	10,362 (Table I)	NA				
11,525 	10,362 (Table I)	VSL				
10,363 	10,218 (Table II)			3P 1C	10C 6R	7R
11,005 	10,218 (Table II)				3P 5C 2R	3C 6R

Biological

Tests for trypanocidal action were made against the Tinde (Tanganyika) strain of *T. rhodesiense*, the Busimbi (Uganda) strain of *T. congolense*, and a South American strain of *T. cruzi*. Mice were infected intraperitoneally (*T. rhodesiense* and *T. congolense*) or subcutaneously (*T. cruzi*) and, when trypanosomes were observed in the blood to the extent of 1 in 2 to 2 in 1 trypanosomes/high power field (1/6 in. objective and a ×6 ocular) of a wet thick blood film, the mice were treated subcutaneously by the injection of 0.5 ml. of a suspension or solution of the compound under test. Infections with *T. rhodesiense* and *T. congolense* were treated with one dose only; *T. cruzi* was given three to five treatments on successive days.

Results

None of the compounds prepared exhibited any action against *T. cruzi* and only slight action at doses approaching the maximum tolerated was obtained against *T. congolense*. Against *T. rhodesiense*, however, several of them showed outstanding activity. The results obtained with this species are listed in Tables I to V. No figures for toxicity are given because this was not done in detail, but in general it can be stated that the arsonic acids (Table I) are less toxic than the oxoarsines (Table II). With the former, doses of 10 mg./20 g. could be given subcutaneously except in the case of 14,373 which killed some mice at 2.5 mg., whereas most of the latter did not permit doses greater than 0.5 mg. The arsenobenzenes shown in Table III appeared to be a little less toxic than the corresponding oxoarsines, and the quaternized compounds in Table IV were possibly more toxic than the unquaternized.

Table V

ACTIVITY AGAINST *T. RHODESIENSE* OF PYRIMIDYL-AMINOPHENYLARSONIC ACIDS LINKED IN THE 2- AND 5-POSITIONS

VSL, see Table I for explanation.

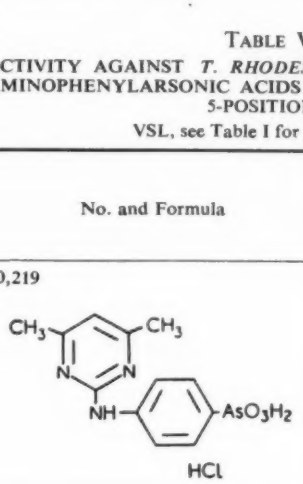
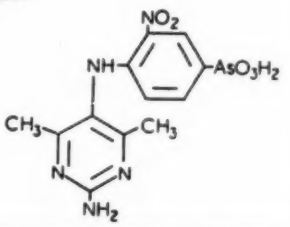
No. and Formula	Results after a Single Subcutaneous Dose (Doses in mg./20 g.)	
	10	2·5
10,219 		VSL
10,364 	VSL	

Table VI

ANALYTICAL DETAILS OF THE COMPOUNDS DESCRIBED IN TABLES I TO III

Ref. No.	Melting Point °C.	Analysis Calculated				Analysis Found			
		C	H	N	As	C	H	N	As
Table I									
10,216	301 (dec.)	35·7	4·1	15·1	20·3	35·7	4·6	14·8	20·3
10,217	331 ,,	32·9	3·8	15·4	20·6	32·9	4·0	15·1	20·7
10,263	293 ,,	—	—	16·3	21·8	—	—	16·4	22·0
10,362	278 ,,	—	—	18·4	19·8	—	—	18·0	19·9
14,373	283–284 (dec.)	37·0	4·4	18·0	19·3	37·1	4·8	17·9	19·3
14,375	280 (dec.)	—	—	18·7	20·0	—	—	18·9	20·2
14,376	268 ,,	34·9	4·2	14·8	19·8	34·7	4·0	14·7	20·0
Table II									
10,185	185–186	33·5	3·4	15·6	20·9	33·7	3·7	14·9	20·7
10,218	200–201 (dec.)	42·9	4·2	18·2	24·3	43·0	3·7	18·3	24·3
11,478	248 ,,	38·0	4·0	11·8	15·8	38·5	3·8	11·5	15·3
11,523	129–130 ,,	37·3	4·4		16·7	36·9	4·5		17·1
12,065 carbonate	273–275 ,,	35·2	4·2	19·5	20·9	35·3	4·0	19·3	20·7
12,065 isethionate	108 ,,	31·8	4·4		16·6	31·4	4·2		16·6
11,524	193–194	37·5	3·4		18·0	37·9	3·6		17·8
14,374	130 (dec.)	43·9	4·6	21·3	22·9	43·6	4·4	21·1	23·0
14,377	100 ,,	40·9	4·3	21·7	23·2	41·3	4·6	21·3	24·0
14,378	110 ,,	40·5	4·6	17·2	23·0	41·6	4·6	17·2	23·0
16,523	195–200 (dec.)	40·8	4·3		23·3	40·3	4·4		23·6
Table III									
10,670	270–275 ,,	38·6	4·5	16·4	21·9	38·7	4·4	16·7	21·8
11,444	260–262 ,,	36·6	4·1		22·9	36·4	4·2		22·9
11,476	224–227 ,,	39·0	3·1	18·2	24·4	38·6	2·8	18·2	24·5
11,477	286–287 ,,	41·6	4·0		26·0	41·2	3·6		26·0

The main conclusions to be drawn from the results listed in the tables are as follows:

(1) The oxoarsines (Table II) are much more active than the corresponding arsonic acids (Table I). The best results were achieved with 12,065 which has an amino group in both the 2- and 4-positions of the pyrimidine ring. Such interference as was made with either of these groups, and particularly the one in the 2-position, lessened activity. The compound 12,065 was selected for further work and it has been shown to be highly effective against infections of *T. rhodesiense* established in the nervous system of monkeys. This work will be reported separately.

(2) The di(pyrimidylamino)arsenobenzenes (Table III) have the same degree of activity as the corresponding oxoarsines, and it is probable that they break down readily to the oxoarsines.

(3) Such quaternizing as was done (Table IV) lessened activity. This is shown particularly when the activity of 10,363 is compared with that of 10,218.

(4) Linking the pyrimidine moiety in the 2- or 5-positions also lessened activity (Table V).

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THE EFFECT OF (+)-LYSERGIC ACID DIETHYLAMIDE AND OTHER DRUGS ON THE CAROTID SINUS REFLEX

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In cats, lysergic acid diethylamide (LSD) selectively blocked the reflex blood pressure rise following carotid chemoreceptor stimulation. It also reduced or abolished the chemoreceptor component of the pressor response to occlusion of the common carotid arteries. It did not inhibit the respiratory reflexes arising from the carotid chemoreceptors, unless spontaneous respiration was interfered with as a whole. The site of action was central, probably below the intercollicular level, regardless of whether the drug was administered by the intravenous route or into the lateral ventricle of the brain.

LSD did not block the baroreceptor depressor reflex elicited by stimulation of one carotid sinus nerve. LSD frequently caused the systemic pressure to fall, even after vagotomy and atropine, and this effect might account for the occasional reduction of the baroreceptor component of the carotid occlusion response. On the other hand, no relationship was found between the action of LSD on vasomotor tone and its blocking effect on the chemoreceptor pressor reflex.

Some derivatives of LSD produced effects similar to those described for LSD, whether or not they possessed a psychotropic action in man, and independently of their efficiency as antagonists to 5-hydroxytryptamine. Of a series of compounds chemically unrelated to LSD, chlorpromazine was found to block the chemoreceptor pressor rise after intracerebroventricular injection.

In two preliminary communications it has been reported that (+)-lysergic acid diethylamide (LSD) is capable of blocking vasomotor reflexes (Ginzel, 1955, 1956). The investigation was particularly concerned with those reflexes which arise from receptors of the carotid sinus region. A detailed account of this work is presented here, including the action of some derivatives of LSD and other substances without chemical relationship to LSD.

METHODS

Fifty cats were used, anaesthetized by intraperitoneal administration of chloralose (70 to 80 mg./kg. body weight). Five experiments were performed using pentobarbitone sodium anaesthesia, and four cats were decerebrated when anaesthetized with chloralose or ether. The blood pressure was measured by means of a mercury manometer connected into one femoral artery. Heparin was injected intravenously in all animals. The trachea was cannulated and, apart from a few experiments where spontaneous respiration was recorded, the animals were respired artificially through an "Ideal" respiration pump. Provision was made to supply the inlet of the pump with either air or pure oxygen.

Both common carotid arteries were dissected free from the surrounding tissues for a length of about 2.5 cm. below the level of the thyroid artery which was cut between ligatures; rubber-covered clamps could therefore easily be applied to the vessels without causing undue stretching. One lingual artery was prepared and a tap cannula was inserted, the tip pointing towards the carotid sinus; through this cannula arterial injections could be made in close proximity to the baro- and chemo-receptors of this region. In order to stimulate the chemoreceptors, small amounts (each dissolved in 0.1 ml. saline) of potassium cyanide, nicotine, or the bis-choline-ester of sebacinic acid (Österreichische Stickstoffwerke A.G., Linz) were injected. The last-named substance is a powerful nicotine-like stimulant drug and is hydrolysed by pseudocholinesterase at a rate similar to that of acetylcholine (Ginzel, Klupp, and Werner, 1952); it is probably because of this high rate of breakdown that it does not show any secondary paralysing effects like nicotine when injected repeatedly in quick succession. It proved, therefore, particularly advantageous when constant responses

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were desired throughout the course of an experiment lasting several hours. During the injections, the external carotid artery was temporarily occluded.

In some experiments, one carotid sinus nerve was dissected and placed on a shielded electrode for electrical stimulation by means of the secondary coil of an inductorium.

Pressor responses were elicited by one of the following procedures: asphyxia produced by clamping the tracheal tube for 60 sec., electrical stimulation of the central end of the cut sciatic nerve, stimulation of one carotid sinus nerve (Neil, Redwood, and Schweitzer, 1949), intra-arterial injection of stimulant drugs to the carotid chemoreceptors and, finally, occlusion of both common carotid arteries for 30 sec. The vagi were sectioned in most experiments in order to avoid activation of homeostatic reflexes from aortic receptors which would tend to counteract these pressor responses.

The substances investigated for their ability to modify the pressor reflexes were administered either intravenously or into the lateral ventricle of the brain through a cannula described by Feldberg and Sherwood (1953). The volume of the injections was kept within 0.1 to 0.2 ml., and regular control injections with saline, adjusted to the *p*H of the test solutions, were given. The position of the intracerebroventricular cannula was checked at the end of each experiment.

In several experiments, intravenous injections of nicotine, adrenaline, and noradrenaline were made before and after LSD, which was administered by either route. The pressor responses to these three drugs were used to test the function of the peripheral pathway at the levels of sympathetic ganglia and effector cells. In two experiments the preganglionic fibres of the superior cervical ganglion were stimulated electrically and the response of the nictitating membrane was recorded.

The following derivatives of (+)-lysergic acid were used in this study: the diethylamide (LSD 25) as the tartrate, its 2-bromo derivative (BOL-148) as the bitartrate, the monoethylamide (LAE 32) and the pyrrolidide (LPD 824) both as the bimaleate, and the (−)-acetyl derivative of LSD 25 (ALD 52) as the bitartrate. The substances, supplied in powder form by Sandoz, were dissolved in distilled water (1:500 or 1:1,000); the solutions were kept in a well-stoppered dark bottle at 0° and they retained their activity for at least a fortnight. 5-Hydroxytryptamine (May and Baker) was used as the creatinine sulphate and chlorpromazine (May and Baker) as the hydrochloride.

RESULTS

The Effect of LSD on Various Vasomotor Responses.—LSD was found to reduce or block rises of the blood pressure which were elicited by the various procedures as described above. The doses ranged between 50 and 200 μg. for the intracerebroventricular injection, and between 50 and 100 μg./kg. body weight when given intravenously. In experiments performed during spring and early summer, the blocking doses of LSD injected intracerebroventricularly were usually around 200 μg. while the doses required in experiments later in the year lay between 50 and 100 μg.

The time of onset of the blocking action of LSD was short, ranging between 1 and 3 min. in different experiments; the maximum effect was reached within 5 to 20 min. Complete recovery was obtained in most experiments between ½ and 1 hr. and the injection of LSD could then be repeated once or twice with comparable results.

The reduction by 200 μg. of LSD in the responses to occlusion of both common carotid arteries and to electrical stimulation of the central stump of the cut sciatic nerve can be seen in Fig. 1. When the reflexes had regained their

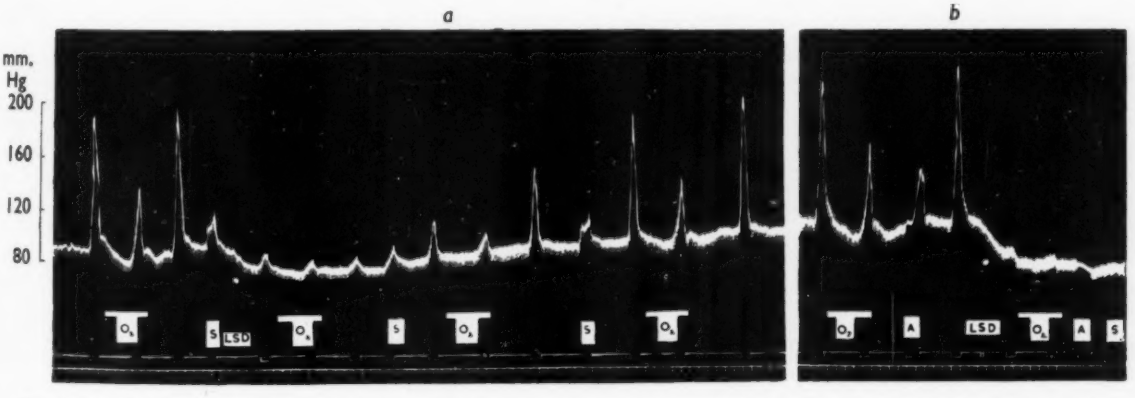

FIG. 1.—Cat, 3.2 kg., chloralose anaesthesia. Both vagi cut, artificial respiration. Records from above down: blood pressure in mm. Hg. The signal alone indicates periods of carotid occlusion; or at S, sciatic nerve stimulation, or at A, asphyxia alone. The horizontal white bars indicate ventilation with oxygen for 2½ min. Time, 30 sec. Between *a* and *b* 10 min. interval. At white dots, injections into the lateral ventricle of the brain of 200 μg. of LSD.

initial size about ½ hr. after the first injection, a second intracerebroventricular injection of the same dose (Fig. 1, *b*) was given and caused a complete block of the pressor responses. This indicated a slight cumulative effect which was, however, not a regular feature. Sometimes a decrease rather than an increase of the blocking action of LSD was observed on repeated administration of the drug. Fig 1, *b* also shows the blocking effect of LSD on the rise of systemic pressure produced by asphyxia.

The effect of LSD on the pressor response to stimulation of the carotid chemoreceptors by intra-arterial injection of sebacinyl-bis-choline (N) is shown in Fig. 2. 150 μg. of LSD, injected into the lateral ventricle, abolished these pressor responses whereas the same dose of LSD previously given intravenously was without effect on the reflex. The chemoreceptor pressor reflex elicited by potassium cyanide was also blocked by LSD.

In the experiment shown in Fig. 2, LSD caused the pressure to fall by about 50 mm. Hg ; more or less (Fig. 1) pronounced depressor effects were seen in other experiments, but occasionally no noticeable change in pressure occurred. The strength of the vasodepressor effects of LSD did not appear to be related to the strength of its blocking action on these reflex responses : thus a small reduction of a reflex could be associated with a great blood pressure fall and the reverse was also true. Moreover, the respective strength of these effects changed sometimes during the course of an experiment. The LSD-induced blood pressure fall was not diminished by vagotomy and atropine.

The actual size of the carotid occlusion reflex depends, at least in part, on the blood pressure level at the time ; any influence, chemical or physical, which lowers the pressure will therefore, of necessity, reduce the response to carotid occlusion.

The Effect of LSD on the Carotid Occlusion Reflex.—Two factors are responsible for the rise in pressure following occlusion of one or both common carotid arteries. One is the reduction in baroreceptor impulses owing to the reduced pressure in the sinus region above the point of clamping ; thus the vasomotor centre, normally inhibited by the baroreceptor impulse traffic, escapes this inhibition and increases its activity. This increase reveals what has been termed the " pressor reserve " and is the greater, the greater the systemic pressure (and consequently the baroreceptor inhibitor tone) was prior to the clamping procedure. The second factor is the increased chemoreceptor impulse activity resulting from the hypoxic condition which develops within the carotid body when the normal blood flow is reduced during clamping (von Euler and Liljestrand, 1943 ; Landgren and Neil, 1951a).

If, therefore, a drug modifies the carotid occlusion reflex (COR) further information as to the nature of this effect should be sought by investigating the two components of the COR separately. This can be achieved when the animal is allowed to breathe air and pure oxygen alternately. Under oxygen, no local hypoxia of the glomus cells develops during the 30 sec. of clamp-

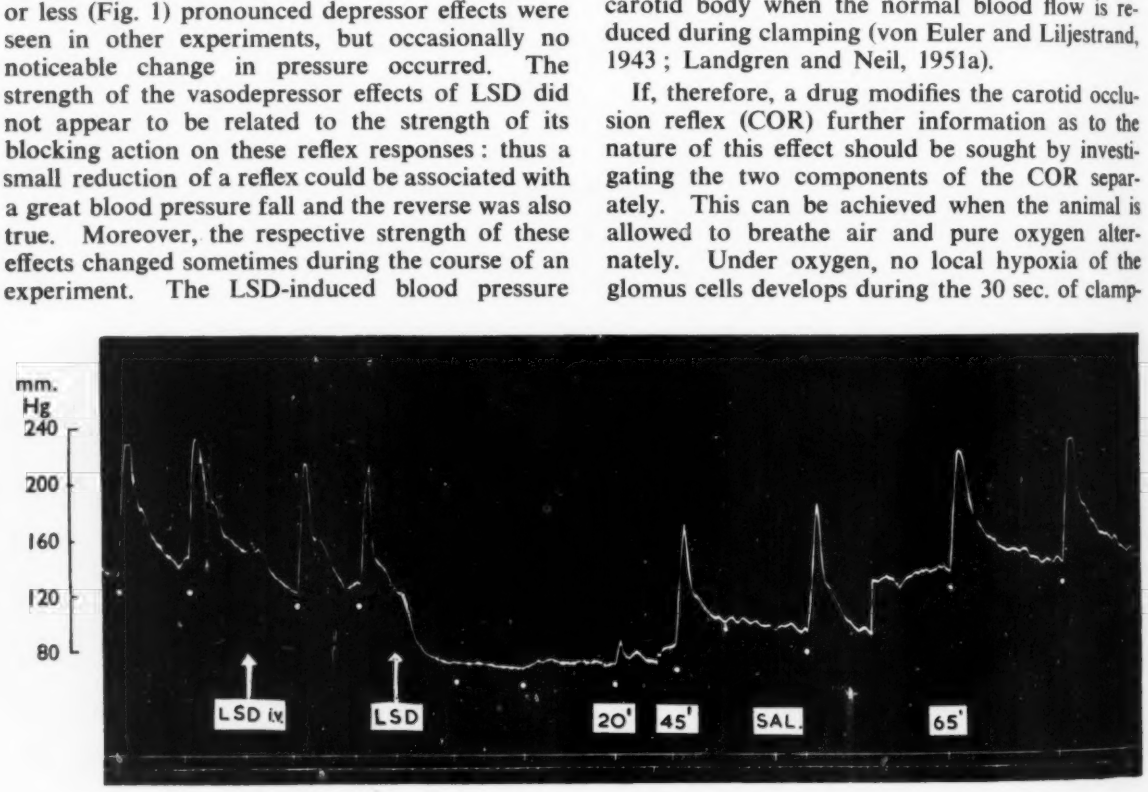

FIG. 2.—Cat, 2.9 kg., chloralose anaesthesia. Both vagi cut, artificial respiration. Traces from above down: blood pressure; injection signal; time, 30 sec. (irregularity in time tracing was due to a mechanical fault in the kymograph). Each white dot indicates injection into the lingual artery of 10 μg. of sebacinyl-bis-choline. Two doses of LSD, each of 150 μg., were given, the first by the intravenous and the second by the intracerebroventricular route. At SAL, 0.1 ml. of saline was injected into the lingual artery. The numerals give the time intervals in min. after the injection of LSD.

ing, the COR being almost entirely baroreceptor in function (Landgren and Neil, 1951b). The height of the COR under air breathing will be increased by the extent to which chemoreceptors contribute to the COR at the time. It will be found that, in the beginning of an experiment with a higher systemic pressure, none or only a small fraction of the COR is due to chemoreceptor activation; towards the end, however, with a gradually falling arterial pressure, the chemoreceptor part of the reflex is increased, whereas that due to changes in baroreceptor activity is diminished. Hence it is conceivable that even at different pressure levels two equally large responses may occur which, in fact, can be shown to be different in respect of the individual size of their contributory factors. It is clear that much caution and criticism are necessary if the effects of a drug on the COR are to be determined.

In Fig. 1, the COR was elicited under air and oxygen breathing respectively. The difference in the height of these reflexes indicated the part played by chemoreceptor activation. After the injection of LSD this difference disappeared; in this experiment, however, the COR had dwindled to such a small residual size that it was not easy to study this particular aspect here. Fig. 3 shows an experiment in which the effects of LSD on the two components of the COR and on the response to N were tested alternately. In this experiment the COR elicited during oxygen breathing was about half as great as that elicited during air breathing. Immediately after the injection of 200 μg. of LSD into the lateral ventricle, the systemic pressure dropped by about 25 mm. Hg and none of the reflex responses could be produced. A few minutes later, when the pressure had regained its initial level, the COR was restored to the height seen during oxygen breathing prior to the injection of LSD. Oxygen breathing reduced the COR now only by about $\frac{1}{6}$, indicating that the chemoreceptor component of the COR was almost abolished. At the same time the chemoreceptor reflex elicited by close arterial injection of N was absent, only reappearing with reappearance of the difference in the height of the COR.

Thus, it is apparent that there exists a stage in the action of LSD where the COR is reduced owing to the abolition of the chemoreceptor contribution. The remaining baroreceptor reflex, however, was also frequently reduced or abolished in the early phase of the effect of LSD, as seen for example in Fig. 3. The reduction might have been simply the consequence of the LSD-induced fall in systemic pressure, which, *per se*, lowers the baroreceptor inhibitor tone and thus diminishes the pressor response during clamping. In the experiment shown in Fig. 1, however, the depressor effect of LSD was very small indeed, whereas the reduction of the baroreceptor component of the COR was quite considerable. In this case, a direct blocking effect of LSD on the synaptic transmission of the baroreceptor afferents must be considered. If such a block developed, a rise in systemic pressure would be expected at the same time. The absence of a rise in pressure could be accounted for by a simultaneous reduction by LSD of central vasomotor tone.

The Effect of LSD on the Baroreceptor Depressor Reflex.—The composite nature of the COR limits its usefulness as a test suitable for investigating the effect of drugs upon the baroreceptor pathway. To permit any conclusion to that end, the baroreceptor depressor reflex should be included in the investigation. The latter can be elicited by electrical stimulation of the carotid sinus nerve; this nerve contains fibres arising both from the baro- and chemo-receptors of the carotid bifurcation area. Depending on the anaesthetic and the type of stimulation (Neil *et al.*, 1949), impulses will predominate in either set of fibres, thus giving rise to a baroreceptor depressor or a chemoreceptor pressor response.

LSD failed to inhibit the blood pressure fall produced by electrical stimulation of one sinus nerve. Fig. 4 shows such an experiment per-

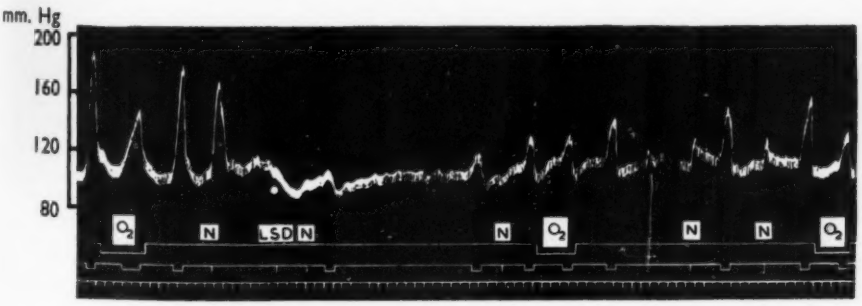

FIG. 3.—Cat, 3.8 kg., chloralose anaesthesia. Both vagi cut, artificial respiration. Tracings from above down: blood pressure; periods of oxygen breathing (O_2); long signal indicates carotid occlusion, brief signal indicates injections of 10 μg. of sebacinyl-bis-choline (N) into the lingual artery and of 200 μg. of LSD into the lateral ventricle. Time, 30 sec.

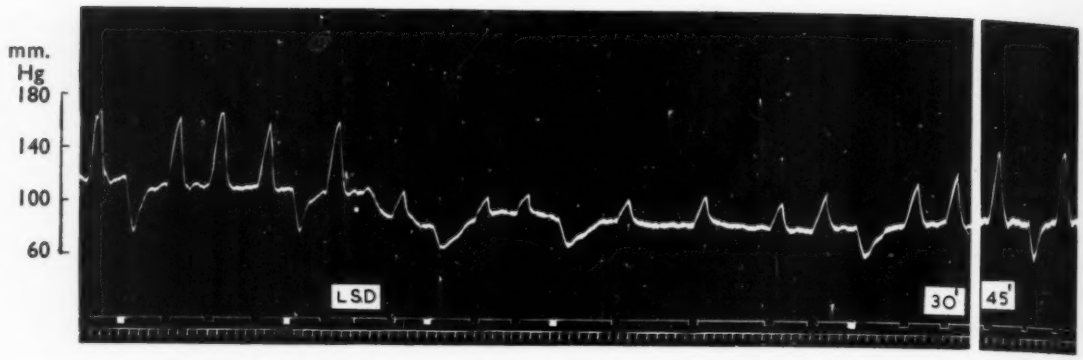

FIG. 4.—Cat, 3 kg., pentobarbitone anaesthesia. Both vagi cut, artificial respiration. Records from above down: blood pressure; long signal indicates carotid occlusion and white blocks indicate stimulation of the left carotid sinus nerve for 20 sec. At the white dot, 150 μg. of LSD was injected intracerebroventricularly. Time, 30 sec. Times after injection of LSD are given.

formed under pentobarbitone anaesthesia where the baroreceptor depressor reflex was elicited in alternation with the COR. About half the size of the latter was due to chemoreceptor activation (oxygen test is not shown). 150 μg. of LSD diminished the COR to less than a third of its original height while sinus nerve stimulation was still as effective as before LSD. The over-all depressor response was somewhat reduced owing to the decreased level of systemic pressure, but the same or even a lower minimum pressure was reached during stimulation. From this it appeared that LSD did not interfere with the baroreceptor pathway when it blocked the chemoreceptor reflex.

This conclusion received further support from an experiment in which sinus nerve stimulation elicited the chemoreceptor pressor reflex (Fig. 5). 50 μg./kg. of LSD was administered, this time by the intravenous route. Shortly after the injection the response to sinus nerve stimulation no longer showed the clear-cut pressure rise but only a series of oscillations suggesting an interplay between reflex pressor and depressor tendencies. Later, these waves were superseded by a distinct depressor effect which increased up to the 25th min. after the injection of LSD. Then it diminished gradually and gave way again to the previous "oscillation" response. After 2 hr. the initial pressor response to sinus nerve stimulation was restored. The effect of noradrenaline, injected intravenously, was not changed throughout the experiment.

The conversion by LSD of the pressor response to stimulation of the sinus nerve into a depressor response suggests that the chemoreceptor impulses which predominated before LSD was given were blocked, and the impulses elicited in baroreceptor fibres during stimulation determined the effect then observed.

When the COR was almost completely inhibited by LSD, it was frequently observed that the pressure dropped below the original level as soon as the clamps were removed from the carotid arteries (see Figs. 3 and 8). This phenomenon was probably nothing more than the carotid depressor reflex set into action by the sudden restoration of

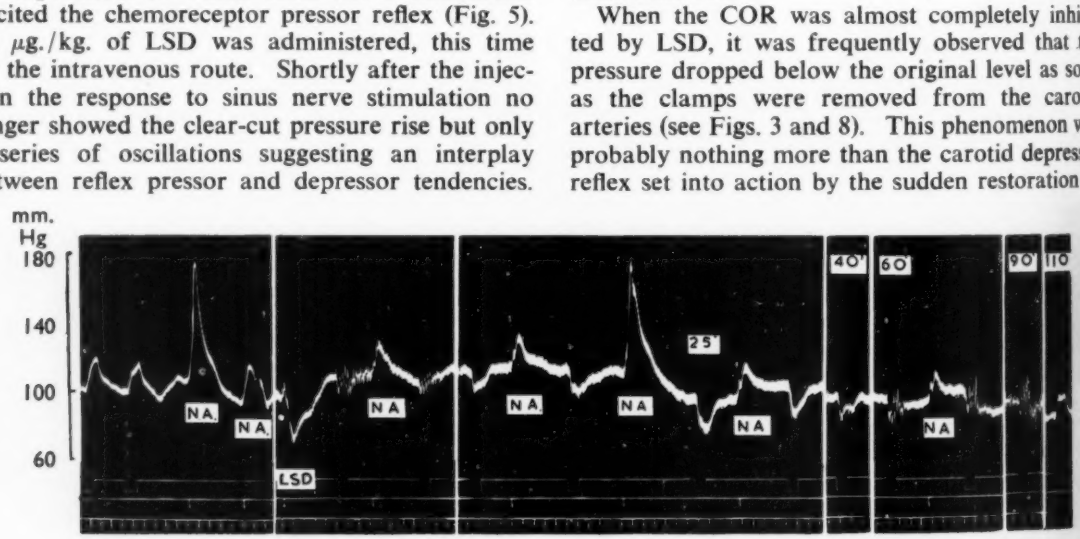

FIG. 5.—Cat, 2.5 kg., chloralose anaesthesia. Both vagi cut, artificial respiration. Tracings from above down: blood pressure; upper signal indicates stimulation of the right carotid sinus nerve; lower signal indicates injection; time, 30 sec. At NA., intravenous injections of 2 and 0.2 μg. of noradrenaline respectively. LSD, 50 μg./kg. LSD intravenously. Times from the injection of LSD are shown.

intrasinusal pressure after declamping. The depressor reflex is usually not visible when the COR develops to full size, then being only part of the general return of the raised pressure to its normal level.

The Site of the Reflex Blocking Effect of LSD.—LSD blocked various vasopressor reflexes after administration of the drug into the lateral ventricle of the brain. The doses found effective by this route in a given experiment were usually without action on the reflex responses when injected into the femoral vein. Somewhat larger quantities administered by the intravenous route, however, did exert a blocking effect.

No reduction of the effects of nicotine, adrenaline, and noradrenaline was observed during and at the maximum of the reflex blockade produced by LSD by either route. When, however, the intravenous dose of LSD was doubled (namely to about 200 μg./kg.) the pressor action of nicotine was sometimes considerably decreased. There was no inhibition of the actions of adrenaline and noradrenaline even after as much as 500 μg./kg. of LSD intravenously. The response of the nictitating membrane to preganglionic sympathetic stimulation was not affected by injection into the lingual artery of 50 and 100 μg. of LSD, indicating that LSD did not impair transmission through the superior cervical ganglion.

From these findings it can be concluded that the block of vasomotor reflexes by LSD did not involve a blockade of impulse transmission through junctional regions within the peripheral efferent pathway of the reflex.

Further, it was found that intra-arterial injection of 50 or even 100 μg. of LSD to the carotid chemo- and baro-receptors did not alter the size of reflexes arising from these receptors. Thus the peripheral part of the afferent reflex pathway also remained unaffected. All this evidence suggests a central point of attack for the blocking action of LSD on vasomotor reflexes.

In an attempt to determine this site of action more closely, two cats anaesthetized with chloralose were decerebrated by suction at the intercollicular level. It was found that LSD blocked the chemoreceptor pressor reflex after this procedure just as it did before. In two cats decerebrated at the same level anaesthetized with ether and then allowed to rest until the anaesthesia had worn off, LSD failed to exert a blocking effect. It did, however, produce a block with the usual dose, after 20 to 30 mg./kg. of chloralose had been injected. Hence, it appears that the ability of LSD to block vasomotor reflexes by interfering with the central part of the reflex pathway below the intercollicular level depended upon the presence of an anaesthetic such as chloralose.

The Effect of LSD on Respiratory Reflexes.—The major physiological significance of the carotid chemoreceptor mechanism lies in its regulatory influence on respiration. A reflex excitation of respiration can be obtained by intra-arterial injections of N or potassium cyanide into the carotid bifurcation. LSD failed to exert a blocking action on this respiratory chemoreceptor reflex.

In Fig. 6 the intracerebroventricular injection of 100 μg. of LSD caused a slowing of respiration ; the response to N, however, was unchanged. A further injection of 100 μg. of LSD reduced the frequency and amplitude of the respiratory movements to a level no longer sufficient for adequate oxygenation, but it still permitted distinct although correspondingly lowered responses to N and KCN. The same behaviour was observed when the respiration was stimulated by the breathing of 5% carbon dioxide. Thus LSD, depressing respiratory function as a whole, lacks a selective blocking action on the respiratory chemoreceptor reflex.

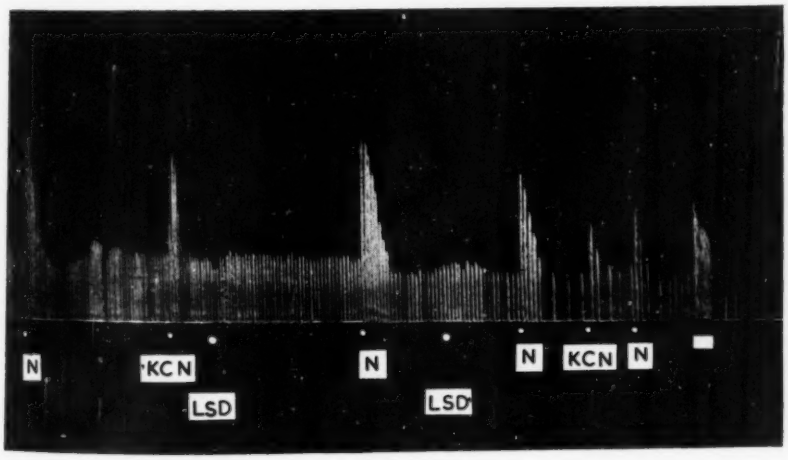

FIG. 6.—Cat, 3.1 kg., chloralose anaesthesia. Both vagi cut. Record of respiration (inspiration only). At white dots, injection into the lingual artery of 8 μg. of sebacinyl-bis-choline at N and 100 μg. of potassium cyanide at KCN. At LSD, intracerebroventricular injections of 150 μg. of LSD. At the white bar, artificial respiration for 30 sec.

The Effect on Carotid Reflexes of Some Derivatives of LSD and of Other Substances.—First of all, it was important to find out whether a derivative without the well-known property to induce autonomic and mental changes in man would or would not exhibit a blocking action on reflexes as LSD. The 2-bromo derivative of LSD, BOL-148, has no psychological action (Cerletti and Rothlin, 1955), but it blocked the COR and the pressor reflex elicited by N, and abolished the difference in the height of the COR during air and oxygen breathing respectively. The strength of the action was about half of that of LSD. Lysergic acid pyrrolidide, LPD 824, which also appears to lack mental actions, was also effective on the reflex; the doses required for intracerebroventricular injection were similar to those of LSD. Of the two psychologically-active derivatives tested, the lysergic acid monoethylamide, LAE 32, possessing only a tenth of the psychotropic potency of LSD, surpassed the reflex blocking effect of the latter by 2 to 5 times whereas the (−)-acetyl lysergic acid diethylamide, ALD 52, did not interfere with reflex responses in doses up to 400 μg. administered intracerebroventricularly.

Dihydroergotamine, another member of the ergot group, was injected into the lateral ventricle; it suppressed the response to chemoreceptor stimulation when given in amounts of at least 300 or 400 μg. The intracerebroventricular injection of 300 μg. of ergotamine (pH of the solution was 4) did not yield a clear result.

Von Euler and Schmiterlöw (1944) have shown that ergotamine, injected intravenously in doses of about 100 μg., inhibited selectively the carotid baroreceptor reflex by specifically inhibiting baroreceptor fibre synaptic transmission at the vasomotor centre. In accordance with their finding it was observed that the part of the COR remaining after the chemoreceptor reflex had been blocked by LSD disappeared promptly when ergotamine was injected. At the same time the baroreceptor depressor reflex could no longer be elicited either.

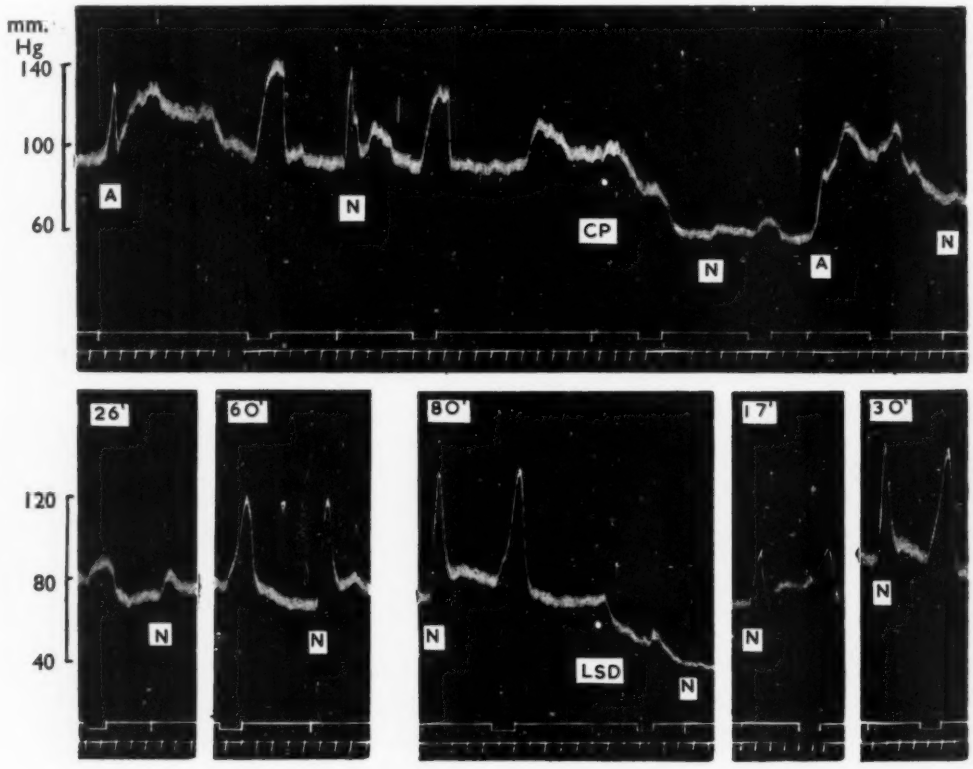

FIG. 7.—Cat, 2.4 kg., chloralose anaesthesia. Both vagi cut, artificial respiration. Tracings from above down: blood pressure; long signal indicates carotid occlusion and brief signal indicates injections. At N, 5 μg. of sebacinyl-bis-choline injected into the lingual artery. At A, 3 μg. of adrenaline intravenously. At CP, intracerebroventricular injection of 200 μg. of chlorpromazine and at LSD of 200 μg. of LSD intracerebroventricularly. Time intervals refer to the intervals elapsing after injections of CP and LSD, respectively. Time, 30 sec.

A number of pharmacologically potent but chemically unrelated substances were also tried in order to determine how common a reflex blocking action was after intracerebroventricular administration of different chemicals. Pentobarbitone sodium, for example, did not interfere with reflex responses even in doses of as much as 6 mg. Mescaline, another drug with psychotropic action, was injected intracerebroventricularly in doses up to 1 mg., but showed none of the actions observed with LSD. Injections of hexamethonium and atropine up to 1 mg. each were without effect. 200 μg. of acetylcholine caused a rise in blood pressure of 40 mm. but did not reduce reflex responses.

LSD is a powerful antagonist to 5-hydroxytryptamine (5-HT) (Gaddum and Hameed, 1954; Ginzel and Kottegoda, 1953). 5-HT was injected into the lateral ventricle in increasing doses; even 1 mg. had no effect whatsoever on carotid reflexes and the blood pressure. Neither did this dose of 5-HT influence the blocking action of LSD, regardless of whether it was administered prior to the injection of LSD or not until the full effect of the latter had developed.

The Effect of Chlorpromazine on the COR.—The last substance in this series tried by the intracerebroventricular route was chlorpromazine (CP). CP had been shown to interfere with vasomotor responses (Dasgupta and Werner, 1954). Kalkoff (1955) and Krause and Schmidtke-Ruhnau (1955) have concluded from their experiments that CP blocks carotid baroreceptor reflexes.

It was found that CP suppressed the response to carotid occlusion and chemoreceptor stimulation when injected intracerebroventricularly in doses of the same order as those of LSD. Fig. 7 shows an experiment where 200 μg. of CP caused such a block; the pressor effect of adrenaline injected intravenously was not affected. One hour after the injection of CP the reflex responses were restored to their original size. A subsequent injection of LSD (200 μg. as well) induced a block again, and once more full recovery was obtained, this time ½ hr. after the injection.

When CP was given by the intravenous route in doses of up to 1.5 mg./kg., it failed to block the chemoreceptor pressor reflex elicited by intra-arterial injection of N. In this way it differed from LSD and from the effect of CP given in to the cerebral ventricles. Still higher doses, amounting to 10 mg./kg., reduced the chemoreceptor response to N by one-third only—namely, by the same extent as they reduced the response to noradrenaline. The action of a previously equipotent dose of adrenaline had already been blocked after the first intravenous injection of 250 μg./kg. of CP.

The fall in systemic pressure following the injection of CP was usually greater than that after LSD. As with LSD, an abolition by CP of the chemoreceptor component of the COR was observed. The carotid depressor reflex elicited by electrical stimulation of the carotid sinus nerve was not inhibited by CP when the COR was blocked (Fig. 8). The COR being abolished, a drop in pressure ensued on removing the clamps from the carotid arteries. These findings agree with those obtained with LSD and permit similar conclusions.

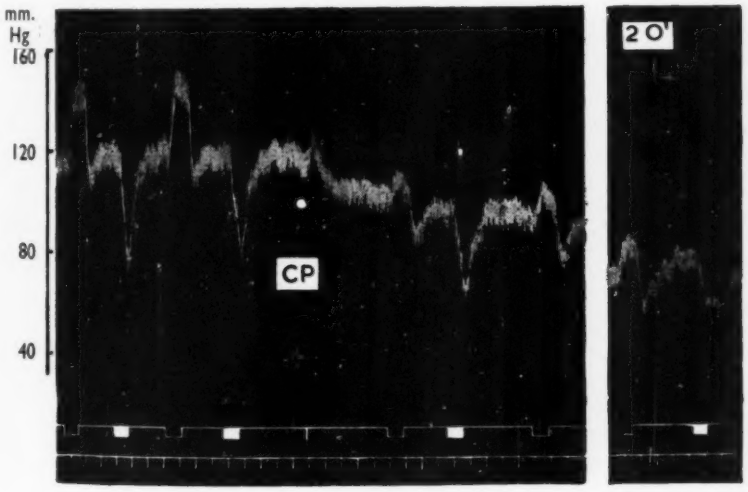

FIG. 8.—Cat, 2.7 kg., chloralose anaesthesia. Both vagi cut, artificial respiration. From above down: blood pressure; long signal indicates carotid occlusion; white bars indicate stimulation of the right carotid sinus nerve; time, 30 sec. At CP, 250 μg. of chlorpromazine was injected intracerebroventricularly.

DISCUSSION

The symptoms produced in man by ingestion of as little as 0.5 to 1 μg./kg. of LSD include disturbances of autonomic functions such as changes in blood pressure and pulse rate, flushing, sweating, trembling, and cold and heat sensations. Seldom is embarrassment of respiration noted.

In anaesthetized cats, LSD was found to interfere with the homeostatic control of blood

pressure based on the function of the carotid receptors, and with other reflexes influencing circulation. The doses required to exert these effects were at least 100 times greater than those used in man. Moreover, the blocking action on vasomotor reflexes was not limited to LSD and those of its derivatives which elicit autonomic and mental symptoms in man. It is therefore unlikely that the results obtained under these experimental conditions have any bearing on the effects which LSD produces in man.

Neither could a direct correlation be established between the blocking effects of LSD and derivatives, and their ability to antagonize 5-HT as tested on the rat uterus. LPD 824 and LAE 32 possessed only 1/20 and 1/8 respectively of the anti-5-HT action of LSD (Cerletti and Konzett, 1956). LPD 824 was equal to LSD in blocking carotid reflexes, whereas LAE was 2 to 5 times stronger. ALD 52 on the other hand, which exceeds the 5-HT-antagonistic potency of LSD, lacked a blocking effect altogether ; it was injected intracerebroventricularly up to a dose twice as large as the blocking dose of LSD.

This comparison, however, does not permit an answer to the question whether the reflex blockade caused by LSD and other substances may or may not involve a mechanism dependent on 5-HT. One has to take into account that the ability of antagonists to block effects elicited by 5-HT varies with the particular test object used (Cerletti and Konzett, 1956). Further, as in the case of the intracerebroventricular administration, the access of these drugs to the site of their action and thus their actual efficacy is eventually determined by the permeability of the CSF-brain barrier. This factor, however, is not known.

On the other hand, little support is lent to the possibility of 5-HT having a rôle in the function of vasomotor reflexes, by the fact that this substance, even in such an exceedingly high amount as 1 mg., interferes neither with these reflexes nor with the blocking effect of LSD. One is justified in assuming that at least a part of the 5-HT has reached the neuronal substrate underlying this action, since smaller amounts of 5-HT can gain access to some other structures of the brain ; Feldberg and Sherwood (1954) have shown that an injection of 75 to 500 μg. of 5-HT into the intracerebroventricular space of a conscious cat provokes definite behavioural and autonomic reactions in the animal.

The fall in blood pressure following the intracerebroventricular administration of LSD or CP still occurred after vagotomy and atropine, and must therefore be due to a diminution of central vasomotor activity. This fall in pressure was sometimes very great with a given dose, but the blocking action on the chemoreceptor pressor reflex was not necessarily great at the same time. On the contrary, it was even observed in two experiments, where the systemic pressure fell to a level as low as 40 mm. Hg, that the response to chemoreceptor stimulation was hardly affected at all ; only a second dose abolished the reflex. The other possible extreme encountered was a very weak effect on the blood pressure with a full block of the chemoreceptor response from the outset. Thus, the effect of these drugs on the reflex appears to be clearly distinct from the effect on the discharge from vasomotor areas. This suggests that each effect has a site of action on a nervous structure afferent in respect to the final relay station (medullary vasomotor centre) in which the efferent vasoconstrictor pathway originates. Alternatively, the efferent neurone of the chemoreceptor pressor reflex might be entirely independent of the final common pathway of sympathetic vasomotor activity.

The results of the investigation into the mechanism by which LSD and CP diminish the blood pressure response to carotid occlusion led to some general conclusions. Frequently it is inferred from a simple reduction of the COR by a drug that neurones underlying the baroreceptor reflexes have been affected. It must be stressed, however, that no such conclusion is permissible unless a pure baroreceptor reflex has actually been tested. The COR is a mixed phenomenon whose chemoreceptor component can differ considerably. A diminution of the COR may sometimes solely be accounted for by the abolition of its chemoreceptor component. This was also the major feature in the action of LSD and CP on the COR. The part of the COR based on baroreceptor function depends on the level of systemic pressure at the time and may therefore decrease merely as a consequence of a decrease in pressure caused by the drug under investigation and not by a direct effect of the latter on the baroreceptor pathway.

The action of LSD on the COR is mainly an action on the chemoreceptor reflex. Ergotamine, however, a substance deriving from the same parent compound as LSD, predominately affects the baroreceptor component of the reflex. Thus, two members of one chemical group are able to distinguish by their action between pathways connecting two different types of peripheral receptors, the chemo- and baro-receptors of the carotid sinus, to one central pool, the medullary vasomotor

centre. On the other hand, with regard to the respiratory chemoreceptor reflex which was not selectively blocked by LSD, a pharmacological differentiation seemed also possible between pathways connecting *one* set of peripheral receptors, the carotid chemoreceptors, with two different central sites, the respiratory and the vasomotor centres of the medulla.

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THE ACTION OF CYSTEAMINE AND SYNKAVIT ON GASTRIC EMPTYING IN THE NORMAL AND IRRADIATED RAT

BY

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A radiographic method for studying the delay in gastric emptying in the rat caused by whole body irradiation has been applied to the investigation of two compounds which modify the effects of irradiation. The method may be of general use in the study of the action of drugs. Synkavit, which can potentiate the lethal effects of irradiation on tumours, caused a delay in gastric emptying. When Synkavit and irradiation were combined, a simple summation of the delays produced by each of these agents occurred. Cysteamine, which gives some protection against the lethal action of *x*-rays, also caused a delay in gastric emptying. It did not protect against the action of *x*-rays on stomach emptying.

X-irradiation gives rise to a delay in gastric emptying in the rat (Goodman, Lewis, and Schuck, 1952; Billings, Bennett, and Burlingame, 1954; Swift, Taketa, and Bond, 1955). The effect of doses as low as 20 r. whole body irradiation (some 3% of the LD50/30 days) has been demonstrated by using serial radiographs to follow the progress of a barium meal in the unanaesthetized rat (Hulse, 1957). Barium meals were used by Smith and Irving (1955) to assess the effect of sodium salicylate on stomach emptying in rats, and it appeared that the routine used in the irradiation experiments was suitable for testing the effect of substances which might alter gastric emptying. Cysteamine [β-mercaptoethylamine] and Synkavit [sodium salt of 2-methylnaphth-1:4-ylene di-(dihydrogen phosphate)] have been tested in this way, cysteamine because it is known to reduce the lethal effects of radiation and Synkavit because it sensitizes tumours to the action of *x*-rays.

METHOD

The details of the technique for giving the barium meal and taking the radiographs have been described elsewhere (Hulse, 1957). The essential points are that the rats were not forcibly fed with the opaque meal and not unduly restrained during radiography. Ease in feeding was achieved by training the rats to take a thick paste of malted milk from a spatula so that when due for experiment they willingly accepted a paste of malted milk mixed with barium sulphate (Horlicks Shadow Meal). A tunnel-like plastic container with opaque sides and one perforated end was used to keep the rat in a suitable position without frightening it. The rat readily entered the container which was closed by sliding a piece of perspex into one of two slots, according to the size of the animal.

Litters of four male rats of an inbred albino strain aged between three and six months were used throughout the experiments. The various treatments given were randomly allocated within the litters.

After a litter had been fed with the barium sulphate mixture, the compound to be tested was given as an intraperitoneal injection to one pair of rats whilst the other pair received the same volume of saline, also given intraperitoneally. The injections were made speedily and gently in order to disturb the rat as little as possible. One animal of each pair was given an appropriate dose of *x*-rays either before or after the compound under investigation was given. The other two rats acted as unirradiated controls.

Radiography

Control radiographs to assess the degree of filling of the stomach at the start of the experiment were taken as soon as possible after the barium meal and before the irradiations. If, as in the case of Synkavit, a period of time had to elapse between administering the test substance and irradiation a further pre-irradiation film was taken. After irradiation, radiographs were taken at half-hourly intervals from the time of irradiation for 3 hr. and then at hourly intervals until 8 hr. had elapsed. During each

radiographic exposure, the rats received a dose of approximately 0.05 r.; the total irradiation through taking radiographs was therefore less than 1.5 r.

Treatments

Synkavit.—Six litters of rats were used. The dose, given immediately after the barium meal, was 1.0 ml. (equivalent to 10 mg. of the free ester). The irradiation (20 r. of *x*-rays) was given half an hour after the administration of the Synkavit.

Cysteamine.—Three different combinations of cysteamine (Becaptan, Labaz) and irradiation were used. With each combination, two parallel series of experiments were performed; in one the cysteamine was given within 5 min. before, and in the other within 5 min. after irradiation. The three dose schemes were 20 mg./kg. of cysteamine and 30 r. of *x*-rays (12 litters), 100 mg./kg. of cysteamine and 20 r. of *x*-rays (eight litters), and 100 mg./kg. of cysteamine and 100 r. of *x*-rays (eight litters).

Radiation Procedures

These were all whole-body irradiations and the irradiation factors were as described previously (Hulse, 1957). All the non-irradiated animals were subjected to sham irradiations.

Results

The results have been assessed in the same manner as in the previous investigation. The degree of fullness of the stomach was judged by comparison with the appearances of the radiograph taken immediately after feeding, and five stages (100, 75, 50, 25, and 10%) were easily distinguished. An average of the degree of fullness of all the rats in an experimental group was obtained and graphs were constructed relating the average degree of fullness to the time after irradiation. In order to compare different treatments, the average of the combined scores from 5 to 8 hr. inclusive after irradiation was calculated for each type of treatment. By subtracting the average of the appropriate controls from that of the experimental groups an expression was obtained for the net effect of each procedure (Tables I and II).

TABLE I

THE EFFECT OF SYNKAVIT ON STOMACH EMPTYING IN THE IRRADIATED RAT

Average fullness of stomach expressed as % at 5 to 8 hr. after irradiation (mean ± S.E.).

Treatment	Observed Effect	Net Effect of Treatment
Saline injection and no irradiation	5±1	—
Saline injection followed by 20 r. of *x*-rays	34±11	29±11
Synkavit injection and no irradiation	42±9	37±9
Sum of net effects of 20 r. saline and Synkavit without irradiation	—	66±14
Synkavit injection followed by 20 r. of *x*-rays	69±9	64±9

Effect of Synkavit.—The injection of 1 ml. of Synkavit by itself gave rise to a delay in gastric emptying slightly greater than 20 r. of *x*-rays, and when Synkavit and 20 r. of *x*-rays were both given a delay in stomach emptying was produced which

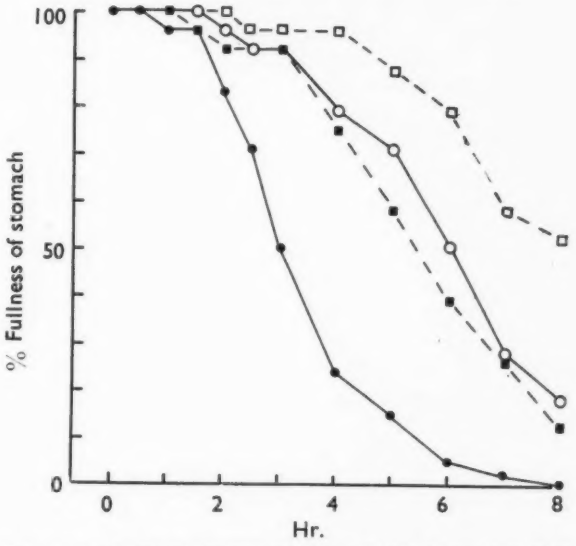

FIG. 1.—The effects of Synkavit and 20 r. of whole body *x*-irradiation on the pattern of gastric emptying. Each curve represents an average of six animals. ——●—— no irradiation, saline injection; — —■— — whole body *x*-rays, saline injection; ——○—— no irradiation, Synkavit injection; — —□— — whole body *x*-rays, Synkavit injection.

TABLE II

THE EFFECT OF CYSTEAMINE AND *X*-IRRADIATION ON STOMACH EMPTYING IN THE RAT

Average fullness of the stomach expressed as % at 5 to 8 hr. after irradiation (mean ± S.E.).

	Observed Effects			Net Effects of Treatments		
Dose of irradiation (r.)	30	20	100	30	20	100
Dose of cysteamine (mg./kg.)	20	100	100	20	100	100
Treatment:						
None (saline injection only)	8±2	12± 2	15±7	—	—	—
X-irradiation (saline injection only)	32±6	33± 5	82±6	24±6	21± 5	67±10
Cysteamine (no saline)	52±5	93± 4	90±4	44±6	81± 4	76± 9
Combined net effects of cysteamine and irradiation	—	—	—	68±9	(102± 7)	(143±13)
Cysteamine followed by *x*-irradiation	81±8	84±12	95±5	73±9	72±1	80± 9
X-irradiation followed by cysteamine	86±4	81±12	95±5	78±5	69±12	80± 9

was greater than either alone (Fig. 1). The net effect of the combined treatment was not significantly different from the sum of the net effects of the treatments given singly ($P>.9$; Table I).

Effect of Cysteamine. — Cysteamine alone caused a delay in gastric emptying, the degree of delay being dependent on the dose. The effect of 100 mg./kg. was very marked (Figs. 3 and 4), whilst that of 20 mg./kg. was less marked but nevertheless quite definite (Fig. 2). During preliminary experiments with various doses of cysteamine it was found that 15 mg./kg. gave a delay only slightly less than 20 mg./kg. and 25 mg./kg. a delay much nearer that of 100 mg./kg.

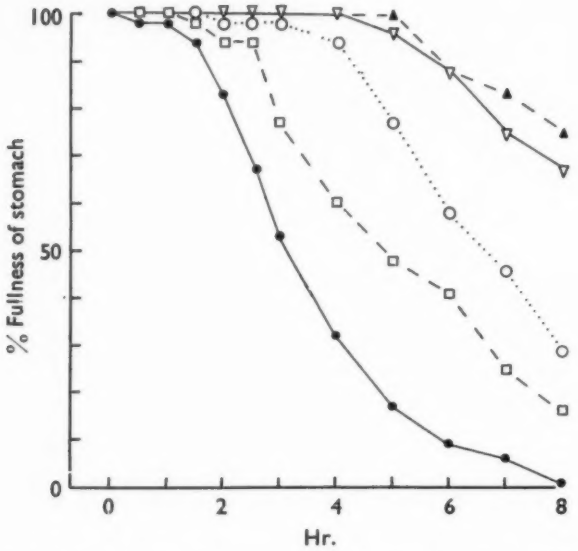

FIG. 2.—The effects of cysteamine (20 mg./kg.) and 30 r. of *x*-rays single and combined (with the cysteamine given either before or after irradiation) on the pattern of gastric emptying. The curves for the animals given no irradiation and saline, 30 r. and saline, and no irradiation and cysteamine are the averages of twelve animals. The curves for cysteamine and irradiation combined are each averages of six animals. —●— No irradiation, saline injection; — — □ — — whole body irradiation, saline injection; · · · · O · · · · no irradiation, cysteamine injection; —▽— whole body irradiation after cysteamine injection; — — ▲ — — whole body irradiation before cysteamine injection.

When 20 mg./kg. of cysteamine was combined with 30 r. of *x*-rays a much greater delay in stomach emptying was produced than either treatment by itself regardless of the order in which the combined treatments were given. The 5 to 8 hr. averages of the combined treatments are almost the same as the sum of the individual treatments (P = .7 to .8 and .3 to .4 ; Table II).

The higher dose of cysteamine (100 mg./kg.) when combined with 20 r. of *x*-rays, regardless of order, gave a delay in gastric emptying much in excess of that produced by 20 r. (Fig. 3) and not significantly different from that of 100 mg./kg. alone (Table II). The combined effects of

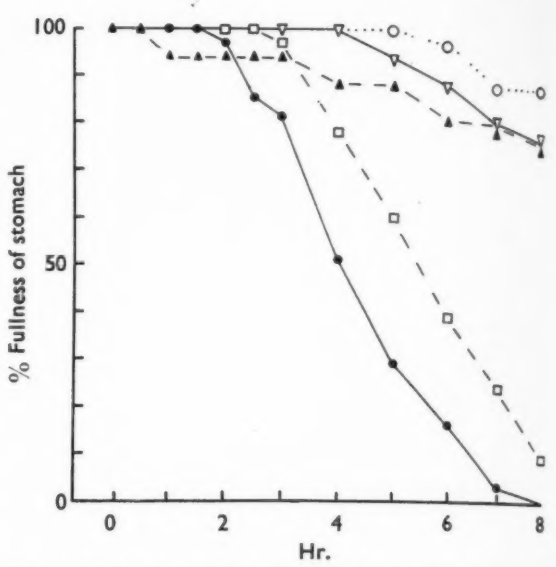

FIG. 3.—The effect on stomach emptying of a high dose of cysteamine (100 mg./kg.) and 20 r. of *x*-rays singly and combined (cysteamine either before or after irradiation). The curves of the cysteamine and irradiation combined both represent averages of four animals. The other curves are each averages of eight animals. —●— No irradiation, saline injection; — — □ — — whole body irradiation, saline injection ; · · · · O · · · · no irradiation, cysteamine injection ; —▽— whole body irradiation after cysteamine injection; — — ▲ — — whole body irradiation before cysteamine injection.

100 mg./kg. of cysteamine and 100 r. of *x*-rays are also independent of the order in which they are given and statistically are similar to the effects of the separate treatments (Fig. 4). The nearly maximal response of the individual treatments using the highest doses prevents a valid comparison between the sum of their net effects and the net effect of the combined treatments (Table II).

Effects of Saline Injections.—An intraperitoneal injection of saline had no appreciable effect on stomach emptying. The previous investigation (Hulse, 1957) included studies of control and irradiated rats which had not received any injections. It is possible to compare these directly with the saline injected animals in the cysteamine experiments. In each case, the shapes of the curves (Figs. 2, 3, and 4) are similar to those obtained previously in the uninjected rat.

During the examination of the radiographs, it was noted that within a few hours of the large dose of cysteamine being given the shape of the

stomach changed and became more globular (Fig. 5). The appearances suggest that there may be a loss of tone in the walls of the stomach after this drug. A tendency towards this change in shape was frequently seen with the smaller dose of cysteamine, but neither Synkavit nor *x*-rays altered the normal contour of the stomach.

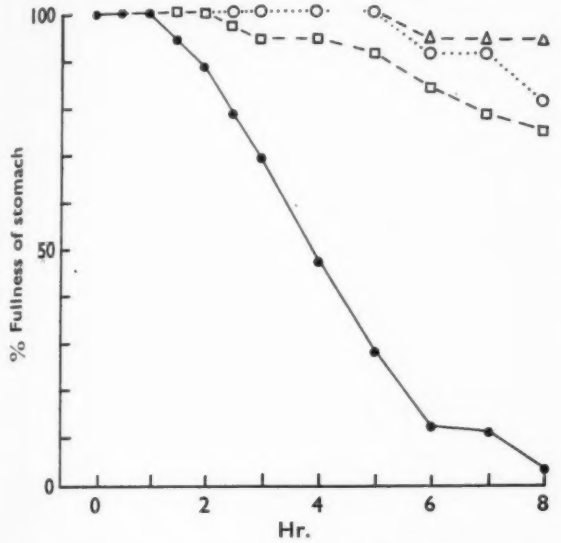

FIG. 4.—The effect on stomach emptying of a high dose of cysteamine (100 mg./kg.) and 100 r. of *x*-rays singly and combined. The curves are each averages of eight animals; exactly similar curves were obtained for the combined treatments whether the cysteamine was given before or after irradiation. —●— No irradiation, saline injection; — — □ — — whole body irradiation, saline injection; · · · · o · · · · no irradiation, cysteamine injection; — — Δ — — whole body irradiation, cysteamine injection.

DISCUSSION

The delay in gastric emptying produced in the rat by ionizing radiations has been amply confirmed since the original observations of Ely and Ross (1947) on neutron irradiation, but the mechanism by which it occurs is still not known. Various other agents are known to give rise to a delay in stomach emptying including muscle ischaemia, traumatic shock, severe haemorrhage, changes in environmental temperature, and injections of sodium salicylate (Whiteley and Green, 1952; Swift *et al.*, 1955; Geiger and Pinsky, 1956; Smith and Irving, 1955). The suggestion that post-irradiation gastric delay is therefore part of a non-specific response to stress (Swift *et al.*, 1955) has been disproved by demonstrating that adrenalectomy does not alter the response (Hulse, 1957). The post-irradiation vomiting seen in other species has recently been studied by neurological methods. Chinn and Wang (1954) found that bilateral destruction of the emetic chemoreceptor zone in the region of the area postrema prevented post-irradiation vomiting in the dog, and Brizzee (1956) confirmed the findings in the monkey and also found that supradiaphragmatic vagotomy inhibited post-irradiation vomiting. Borison (1957) has extended this type of work, using cats as the experimental animal, and concluded that the phenomenon is initiated in the abdomen and that the afferent impulses traverse both the vagus and the dorsal spinal roots, and that the segmental pathway involves innervation which is independent of the sympathetic distribution.

Synkavit has been used experimentally and therapeutically as a means of enhancing the effects of irradiation on tumours (Mitchell, 1954, 1955). The results reported here show that Synkavit has a similar action on stomach emptying to that of irradiation. When irradiation and Synkavit were combined the result was a simple summation of their individual effects (Table I).

Cysteamine, when given before irradiation, reduces the numbers of deaths occurring in groups of experimental animals during the first few weeks after exposure, but when given after irradiation has no effect (Bacq and Alexander, 1955). The drug has, however, been given after therapeutic irradiation in man to combat the symptoms of radiation sickness (Bacq and Herve, 1952; Bacq, 1953, 1954; Bacq, Dechamps, Fischer, Herve, Le Bihan, Lecomte, Pirotte, and Rayet, 1953), but Court Brown (1955) was unable to confirm the beneficial results which had been reported. In the present experiments cysteamine has been found to give a delay in gastric emptying in the rat similar to that produced by irradiation itself. With none of the schemes of dosage and with neither of the orders of treatment was there any reduction of the irradiation effect when the irradiated animal was treated with cysteamine, in fact the evidence points to the two delays being additive (Table II). As rats are unable to vomit, gastric delay may be analogous to post-irradiation vomiting seen in man. If so the present results offer support to the contention of Court Brown that cysteamine has no therapeutic action in radiation sickness.

A widely accepted explanation for the action of cysteamine in reducing the lethal effects of *x*-rays is that it competes for the free radicals produced by radiation and is itself oxidized in the process. As both irradiation and cysteamine cause a delay in gastric emptying it would be expected that any such antagonistic action would be at a maximum when the magnitude of the delay caused by the

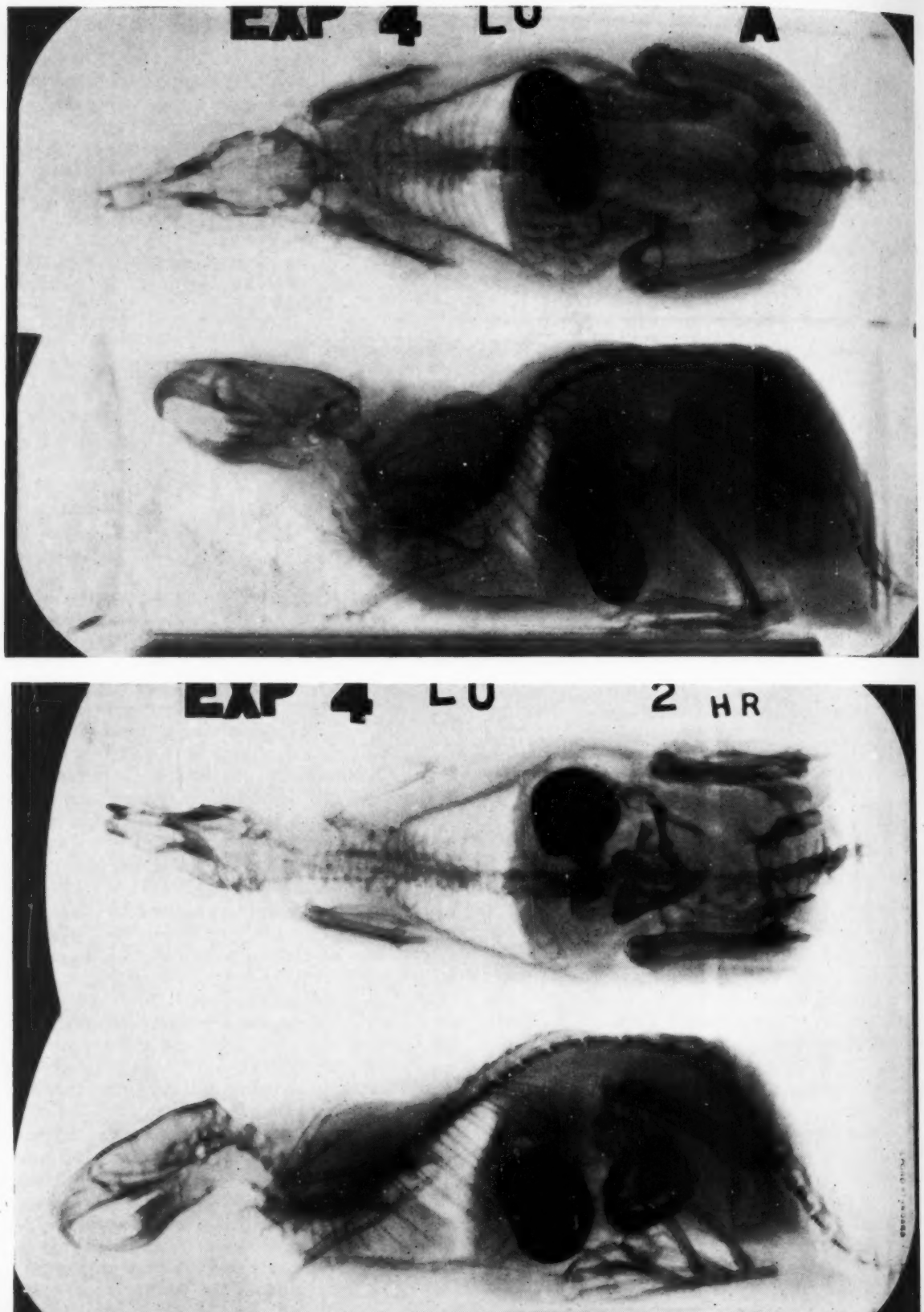

FIG. 5.—Lateral view radiographs of a rat stomach (*a*) just before and (*b*) 2 hr. after receiving 100 mg./kg. of cysteamine. No appreciable emptying of the stomach had taken place, but after cysteamine the stomach has become globular in shape.

cysteamine and the irradiation was about equal. The doses of 30 r. of *x*-rays and 20 mg./kg. of cysteamine were chosen with this in view, but the two agents are in this case clearly additive in their effects. Although these results are not sufficient to dogmatize upon, they do at least suggest either that delay in gastric emptying is not initiated by the action of free radicals or that the theory of free radical competition is inapplicable in the case of gastric emptying as presumably it is in the case of certain genetic effects of irradiation (Kaplan and Lyon, 1953a and b).

It has been suggested that cysteamine does not reduce the apparent radiation damage by interfering with the initial interaction between radiation and living tissues but affects, by some biochemical mechanism, the biological repair process (Bond and Cronkite, 1957). No evidence, however, for such an action was found in the case of radiation injury of the intestinal epithelium (Mole and Temple, 1957). If, as is quite probable, recovery from radiation-induced gastric delay is represented by the stomach emptying which eventually takes place, it is clear that from this aspect also cysteamine offers no protection.

Given proper precautions, of which gentle handling and training of the rats are probably the most important, serial radiographs after a barium meal can be a simple method of investigating agents which may effect stomach emptying. The method may be particularly useful for testing compounds designed to alleviate post-irradiation nausea and vomiting. Other methods of making numerical comparisons can be used instead of the 5 to 8 hr. average. For instance the time taken for the stomach to reach half-full (50% emptying time) is a possible measure if the time of observation is extended to well over 8 hr. Care, however, would have to be taken that the dose of irradiation from taking the larger number of radiographs was not excessive. Whenever this method of studying stomach emptying time is used, the sensitivity of the system to irradiation makes it important that a check should be made on the amount of irradiation received during radiography and precludes the use of screening.

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THE EFFECT OF COOLING AND OF 5-HYDROXYTRYPTAMINE ON THE PERISTALTIC REFLEX OF THE ISOLATED GUINEA-PIG ILEUM

BY

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Cooling the guinea-pig ileum to 19 to 26° abolished the emptying phase of the peristaltic reflex. The effects of cooling to 10° were usually reversible, but cooling to 5° for 3 to 8 hr. produced an irreversible decrease in or abolition of the emptying phase. 5-Hydroxytryptamine (5-HT) added in low concentrations to the fluid outside the intestine restored slight peristaltic activity after this activity had been abolished by cooling. If the peristalsis had been depressed but not abolished by cooling, 5-HT in the bath sometimes abolished it. 5-HT introduced into the lumen of the cooled gut regularly restored or increased peristaltic activity. This action was prevented by previous injection of 2-bromolysergic acid diethylamide into the lumen. It is suggested that, when introduced into the lumen, 5-HT may sensitize the sensory receptors in the mucosa of the cooled intestine. When applied outside, 5-HT may facilitate transmission at synapses involved in the peristaltic reflex arc, the excitability of which has been depressed by cooling.

It was found in previous work on the isolated rabbit colon that cooling the preparation had a greater effect on synaptic transmission than on depolarization of the postsynaptic neurone or on conduction in the postganglionic fibres (Varagić and Beleslin, 1957). Bülbring and Lin (1957) suggested that the formation of 5-hydroxytryptamine (5-HT) by the intestinal mucosa may be part of the physiological mechanism of peristalsis; 5-HT is also known to depress or abolish the peristaltic reflex (Kosterlitz and Robinson, 1957; Ginzel, 1957). We have therefore studied the effect on the peristaltic reflex of cooling and of 5-HT on the cooled intestine. It was hoped that these experiments might provide additional information about the physiological rôle of 5-HT in peristalsis.

METHODS

A modification of the method of Trendelenburg (1917) was used. The apparatus was arranged as shown in Fig. 1. The upper end of a piece of guinea-pig ileum (G) was tied to a small glass cylindrical cap with a side-piece on which a rubber membrane was fitted (A). Through this it was possible to introduce the drug into the lumen of the intestine; it could be washed out through another side-piece (B). The glass cap was attached to a lever to record longitudinal muscle contractions. A record of peristaltic activity was made with the volume recorder described by Stephenson (1948). The peristaltic reflex was tested by raising the intraluminal pressure to a constant height (varying from 20 to 35 mm. in different experiments) for 60 sec. at constant intervals.

The gut was cooled by lowering the temperature of the bath. The intraluminal injections were made in 0.2 ml. volume. The intestine was bathed in Tyrode solution through which a gas mixture containing 97% O_2 and 3% CO_2 was bubbled at a constant rate throughout the period of cooling. Unless otherwise stated the volume of the bath was 20 ml.

The drugs used were 5-hydroxytryptamine creatinine sulphate and 2-bromolysergic acid diethylamide (BOL-148).

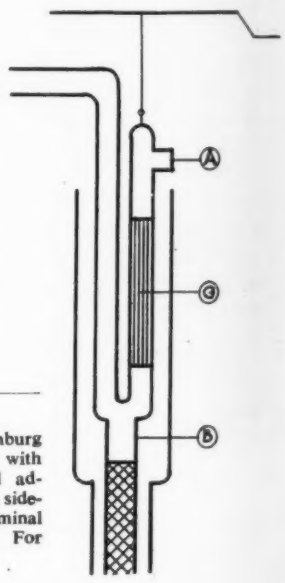

FIG. 1.—Modified Trendelenburg preparation. A, glass cap with side-arm for intraluminal administration of drugs. B, side-arm for removal of intraluminal fluid. G, guinea-pig ileum. For fuller explanation see text.

RESULTS

The Effect of Cooling on the Peristaltic Reflex.—It is known that, in intestinal preparations which have been stored at 5° for 18 to 24 hr., only contraction of longitudinal muscle (preparatory phase of the peristaltic reflex) can be elicited (Ambache, 1946). We found that lowering the bath temperature to 19 to 26° abolished the emptying phase of the peristaltic reflex, and that cooling the preparation to 5° for 3 to 8 hr. caused irreversible changes in the gut in consequence of which the emptying phase was substantially reduced or abolished.

In 8 out of 20 experiments the emptying phase was abolished at 25°, and in 6 out of 20 at 24°. In 5 experiments in which the bath temperature was lowered to 26 to 27° the peristaltic reflex was only slightly depressed. The rate and amplitude of contraction of the circular muscle were diminished.

In some experiments the effects of cooling to 10° were reversible if sufficient time was allowed for the preparation to recover after rewarming. In other experiments after only moderate cooling (to 24°) the peristaltic reflex was not completely restored after rewarming the gut.

A typical experiment is shown in Fig. 2. Between *a* and *b* the preparation was cooled to 5° for 3 hr. The temperature of the bath was then raised to 37° and a period of 1 hr. was allowed for the preparation to recover. At the end of this period (Fig. 2*b*), raising the intraluminal pressure produced very slight peristaltic activity. The emptying phase was greatly reduced but the preparatory phase was almost unchanged.

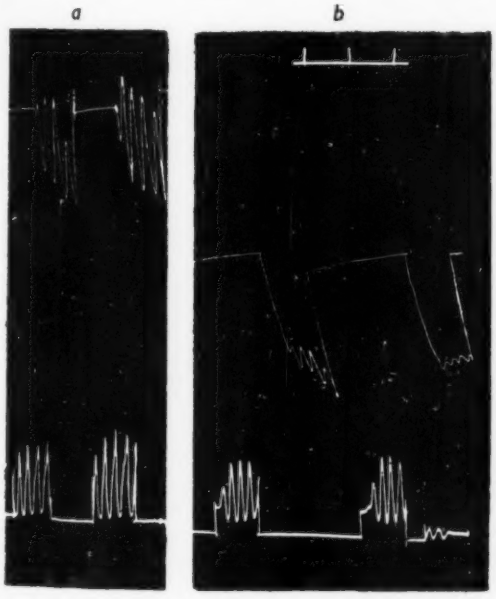

FIG. 2.—Effect of cooling on peristaltic activity of isolated guinea-pig ileum. Upper record, peristalsis. Lower record, contractions of the longitudinal muscle. *a*, normal reflex. Between *a* and *b*, bath temperature 5° for 3 hr. *b*, 1 hr. after rewarming to 37°. Time, 1 min.

The Effect of 5-Hydroxytryptamine (5-HT).—Koelterlitz and Robinson (1957) and Ginzel (1957) reported that 5-HT depressed or abolished the peristaltic reflex of the guinea-pig ileum when added to the bath in concentrations of 10^{-6} to 10^{-5} g./ml.; in these experiments the 5-HT was acting on the outside of the intestine. In the present experiments the addition of 5-HT to the bath in concentrations of 5×10^{-9} to 2×10^{-8} g./ml. caused the reappearance of weak peristaltic activity after this activity had been abolished by cooling. In the experiment illustrated in Fig. 3, lowering the bath temperature to 25° between *a* and *b* abolished the emptying phase of the peristaltic reflex. After the addition between *b* and *c* of 0.4 μg. 5-HT to the bath, making the final concentration 2×10^{-8} g./ml., slight peristaltic activity could be produced. When a similar dose of 5-HT was added to the bath 12 min. later, between *c* and *d*, the peristaltic reflex could not be elicited. When this dose was introduced into the lumen of the intestine, peristalsis appeared again when the pressure was raised, as shown in *e*. The dose of 0.4 μg. of 5-HT was injected in 0.2 ml., and subsequent filling of the intestine produced an internal concentration which lay between 1 and 2×10^{-7}.

These effects were observed in 11 of 13 experiments in which the gut was cooled to 24 to 27°. In the other two experiments the cooling caused only a decrease in the number and amplitude of the peristaltic waves. The addition of 5-HT to the bath caused an initial slight increase and a subsequent abolition of the peristaltic reflex. The preparation, whose activity was recorded in Fig. 4, was cooled between *a* and *b*. The effect of 5-HT, 4×10^{-8} g./ml., is shown in *c*. Between *c* and *d*, the 5-HT was allowed to act for 20 min., then washed out, and the preparation was left for 2 hr. The peristaltic reflex was still abolished (Fig. 3*d*). However, after the introduction of a similar dose of 5-HT into the lumen of the intestine between *d* and *e* the peristaltic reflex was restored.

In another series of 6 experiments the bath was cooled to 20 to 25°; this caused complete abolition of the peristaltic reflex. The addition of 5-HT to the bath in concentrations of 5×10^{-9} to 5×10^{-8} g./ml. did not cause the reappearance of peristalsis in these experiments.

The introduction of 5-HT into the lumen of the guinea-pig ileum in doses of 10 to 400 ng., after peristalsis had been abolished by cooling, regularly caused the re-appearance of peristalsis. This was in accordance with the findings of Bülbring and Lin (1957), and was observed in 10 experiments. In 2 experiments, 5 ng. of 5-HT introduced into the lumen was found to be insufficient to produce peristalsis.

Effect of 2-Bromolysergic Acid Diethylamide (BOL-148).—Fig. 5 illustrates the abolition of the effect of 5-HT by BOL-148. Lowering the bath temperature to 24° abolished the peristaltic reflex (Fig. 5*b*, 1). The addition of 0.1 μg. 5-HT to the bath, making the concentration 10^{-8} g./ml., did not produce any visible change (2). After the introduction of 5 ng. 5-HT into the lumen, raising the pressure produced three peristaltic waves (3). Between *b* and *c*, BOL-148 was left in the lumen for 5 min. The introduction of 5-HT into the lumen while BOL-148 was present caused no reappearance of peristalsis (Fig. 5*c*).

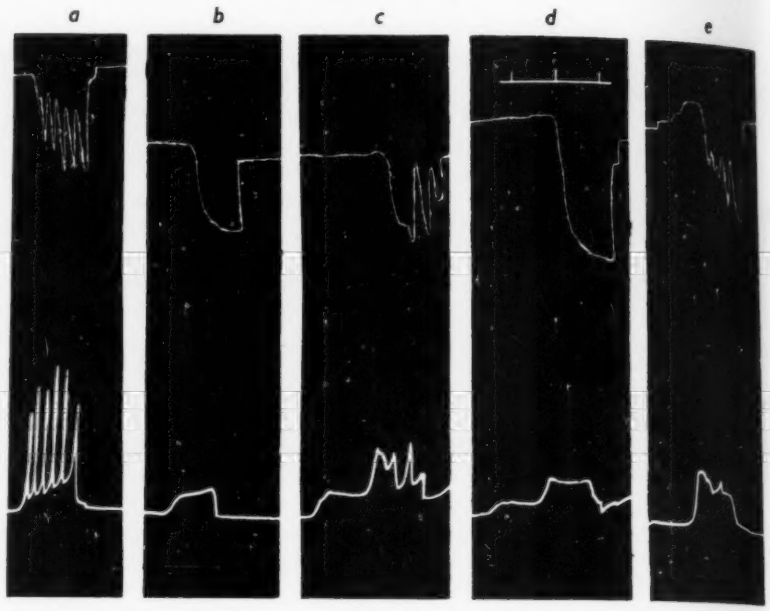

FIG. 3.—Effect of 5-HT on cooled guinea-pig ileum. Upper record, peristalsis. Lower record, contractions of longitudinal muscle. *a*, normal reflex at 37°. Temperature after this point, 25°. *b*, reflex at 25°. *c*, 0.4 μg. 5-HT added to bath. *d*, similar dose 12 min. later. *e*, similar dose introduced into lumen of gut. Time, 1 min.

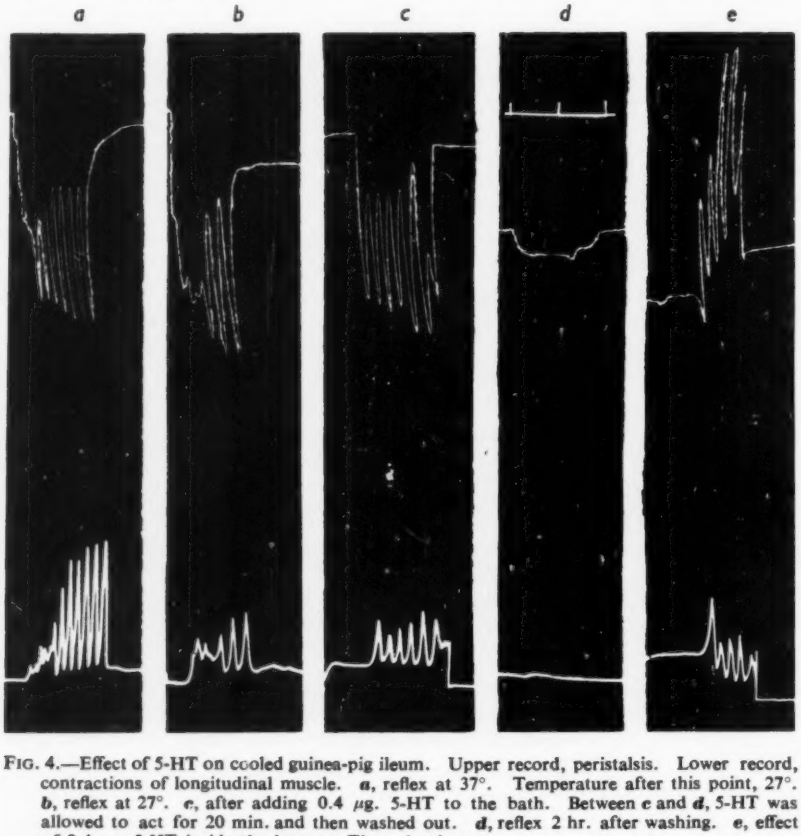

FIG. 4.—Effect of 5-HT on cooled guinea-pig ileum. Upper record, peristalsis. Lower record, contractions of longitudinal muscle. *a*, reflex at 37°. Temperature after this point, 27°. *b*, reflex at 27°. *c*, after adding 0.4 μg. 5-HT to the bath. Between *c* and *d*, 5-HT was allowed to act for 20 min. and then washed out. *d*, reflex 2 hr. after washing. *e*, effect of 0.4 μg. 5-HT inside the lumen. Time, 1 min.

DISCUSSION

In the present experiments, cooling the guinea-pig ileum to temperatures between 19° and 26° abolished the emptying phase of the peristaltic reflex. In the majority of experiments this abolition took place at 24 to 25°. Feldberg and Lin (1949) observed incomplete inhibition of the emptying phase at 32.5°, while Kosterlitz and Robinson (1957) observed

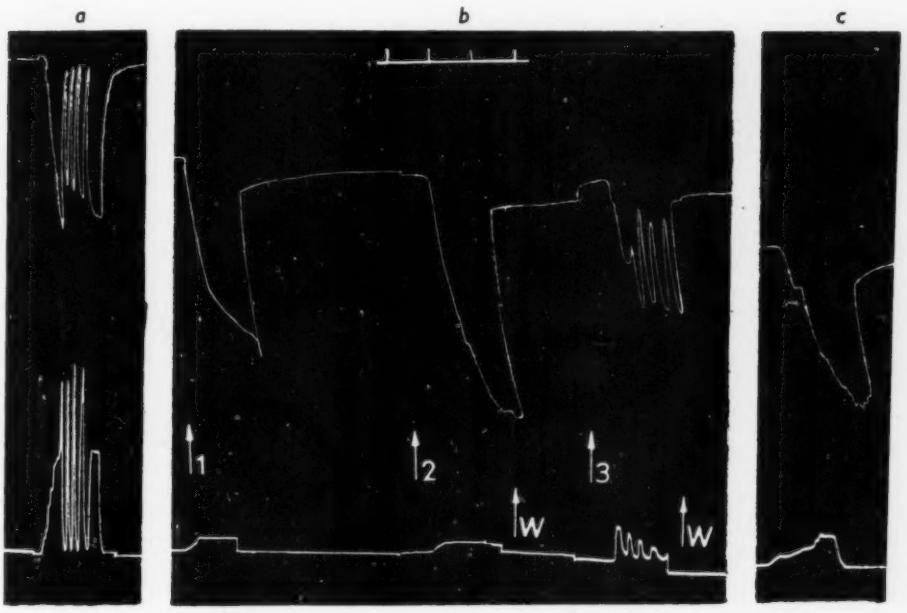

FIG. 5.—Effects of 5-HT and BOL-148 on cooled guinea-pig ileum. *a*, reflex at 37°. Temperature after this point, 24°. *b*, no addition to bath. 1, reflex. 2, 0.1 μg. 5-HT added to bath. 3, 5 ng. 5-HT intraluminally. Between *b* and *c* 0.2 μg. BOL-148 was left in the lumen for 5 min. *c*, 5 ng. 5-HT intraluminally in the presence of BOL-148. Time, 1 min.

that the co-ordinated contractions of the circular muscle layer ceased at 21°. In the present experiments it was found that the effects of cooling to temperatures between 27° and 10° were usually reversible. On the other hand cooling to 5° even for short periods (3 to 8 hr.) caused irreversible changes, and reduced or abolished the emptying phase of the peristaltic reflex. The cooling seldom affected the contractions of the longitudinal muscle.

5-HT added to the bath at 37° in concentrations from 10^{-6} to 10^{-5} g./ml. depresses or abolishes the peristaltic reflex (Kosterlitz and Robinson, 1957; Ginzel, 1957). The present experiments show that 5-HT added to the bath in concentrations from 5×10^{-9} to 2×10^{-8} g./ml. may cause reappearance of slight peristaltic activity in preparations which have been cooled to 24 to 25°. This was evident when the peristaltic reflex had been abolished and also when it had only been depressed by the cooling. It was particularly easy to restore peristalsis in the cooled guinea-pig ileum with 5-HT by introducing the drug into the lumen in doses of 10 to 400 ng.

There are at least three possible explanations of the effect of 5-HT on the cooled guinea-pig ileum: (*a*) it may act by sensitizing the sensory receptors in the mucosa which trigger the reflex, as suggested by Bülbring and Lin (1957); (*b*) it may facilitate the transmission of impulses from afferent to efferent parts of the reflex arc; or (*c*) it may act in both ways, according to the site of application. In favour of the second hypothesis (*b*) is the finding of Trendelenburg (1956) that 5-HT potentiates the response of the nictitating membrane to submaximal preganglionic stimulation of the cervical sympathetic chain. It has also been shown that synaptic transmission in the isolated rabbit colon is more easily blocked by cooling than is depolarization of the postsynaptic neurone or conduction in the postganglionic fibres (Varagić and Beleslin, 1957). Morphological investigations (Bülbring, Lin, and Schofield, 1958) suggest that there is at least one synapse in the peristaltic reflex arc. This is also supported by the fact that this reflex may be abolished by large doses of nicotine (Feldberg and Lin, 1949) and by methonium compounds (Paton and Zaimis, 1949). It is therefore possible that 5-HT may act by facilitating transmission at a synapse of the peristaltic reflex arc. Thus, while 5-HT at normal temperature prevents the peristaltic reflex, it appears to be able to exert the opposite effect when the excitability is depressed under the special conditions of cooling.

In favour of the first hypothesis is the finding that 5-HT is most effective in restoring the peristaltic reflex after cooling when it is introduced into the lumen. In addition, we found that, both in the cooled guinea-pig ileum (Fig. 4) and in the ileum at 37°, the intraluminal injection of 5-HT sometimes restores slight peristaltic activity when

this has been prevented by a large dose of 5-HT acting on the outside of the intestine. In these conditions, the stimulation of the sensory receptors in the mucosa by intraluminal 5-HT may increase the impulses going to the effector cells and thereby overcome the block.

It is concluded that applications of 5-HT to the outside may have a directly facilitating action on ganglionic transmission in the cooled intestine, which is not seen in normal conditions. The intraluminal application of 5-HT, on the other hand, may relieve ganglionic block in an indirect way, by augmenting sensory impulses arising from mucosal receptors.

We are indebted to Dr. Edith Bülbring, of the Department of Pharmacology, Oxford, for criticizing the manuscript, and to Mr. Ivan Gašpar for technical assistance. 5-Hydroxytryptamine creatinine sulphate was kindly supplied by S. A. Farmaceutici, Milano, and 2-bromolysergic acid diethylamide by Sandoz A.G., Basel.

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SLOW-CONTRACTING SUBSTANCES IN HUMAN URINE

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A method has been developed for purifying the principle in human urine causing contraction of the rat uterus. The method, based on adsorption on the cation exchange resin Amberlite IRC-50, is better suited for dealing with large quantities of urine than the method of Gomes (1955). The presence of the two active fractions described by Walaszek (1957) has been confirmed by a different method.

The stimulating effect of normal urine on isolated smooth muscles has been investigated by Werle and Erdös (1954), Gomes (1955), and Walaszek (1957), who found evidence of a substance having the properties of a polypeptide closely related to bradykinin, and consequently to the group of substances consisting of kallidin, bradykinin, and substance Z (Gaddum, 1955). Walaszek (1957) purified further the active principle in urine extracts prepared according to Gomes (1955) and found two substances having a contracting effect on the rat uterus.

In the present paper, a method is described for purifying the rat-uterus-stimulating component in human urine and a procedure described by which the active principle could be divided into two fractions. The technique of separation differs from that of Walaszek (1957), because using his method none of his substance Z_1 could be recovered and only small quantities of substance Z_2.

METHODS

Assay.—Uteri from virgin rats weighing 140 to 180 g. were superfused with de Jalon solution (Gaddum, Peart and Vogt, 1949) by the technique described by Gaddum (1953), or were suspended in 3 ml. baths. The rats were injected with stilbœstrol (10 μg./100 g.) on the day before the assay. The period of contact of active solution was for 30 sec. at intervals of 5 min. Atropine sulphate (10^{-6}) was added to the de Jalon solution.

Aliquots of the fractions from the chromatographic columns were generally freeze-dried prior to the assay. Extracts in water, however, were usually diluted with an equal volume of double-strength de Jalon solution; the hydrochloric acid fractions were occasionally titrated with sodium hydroxide solution, and de Jalon solution in double concentration was then added, but with a calculated smaller content of sodium chloride. Organic solvent was removed from fractions containing aqueous methanol or acetone *in vacuo* at room temperature, and the remainder thereafter lyophilized.

Urine Extracts.—Urine extracts were prepared from 24 hr. urine specimens by a butanol extraction method described by Gomes (1955) and later employed by Walaszek (1957). Each preparation yielded from 1.2 to 1.7 g. of extract and the dry extracts appeared to be stable for at least 10 months.

Standard Preparation.—The first urine extract produced was used as standard, one unit having been taken as the activity in 1 mg. of this extract.

Stability Tests at Different pH Values.—The stability of urine extracts was tested within the *p*H range of 0 to 14. For *p*H values from 0 to 3 hydrochloric acid was used and from 12 to 14 sodium hydroxide; from *p*H 3 to 7 the solutions were buffered with citric acid and disodium hydrogen phosphate, from *p*H 7 to 10 with boric acid and sodium hydroxide and from *p*H 10 to 12 with disodium hydrogen phosphate and sodium hydroxide. The *p*H values were determined with a glass electrode.

The *p*H values of the urine extracts in distilled water ranged between 3.3 and 3.8, and the solutions had some buffering capacity. In the stability tests, it was therefore necessary to use added buffers in such amounts as to overcome the buffering capacity of the extracts. Care was taken, however, that the amounts were not so large as to interfere with the uterine contractions after the dilution with de Jalon solution. Extracts containing hydrochloric acid or sodium hydroxide were neutralized with sodium hydroxide, or hydrochloric acid, prior to the assay.

Dialysis.—Cellophane casings (Visking Corp., Chicago, Illinois) were used.

Paper Pulp Chromatography.—Whatman cellulose powder was used. As a rule, the columns were built up with paper pulp suspended in 0.2 N-hydrochloric acid. After the paper had settled down, it was

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usually washed thoroughly with 0.2 N-hydrochloric acid, then washed free of acid with distilled water, and finally washed with the fluid to be used as solvent for the active substance at the succeeding chromatography. In a few experiments the columns were intentionally *not* washed in advance with hydrochloric acid.

Aluminium Oxide Chromatography.—Three types of aluminium oxide (Woelm, Eschwege, Germany) were used, an alkaline, a neutral, and a weakly acid preparation.

Ion Exchange Chromatography.—Amberlite IRC-50 (analytical grade) was used to adsorb the active substances from urine. The resin was prepared in the following way. 100 g. of Amberlite IRC-50 (40 to 60 mesh) in the hydrogen form was stirred with 350 ml. of 1 N-HCl for 30 min. and then washed by decantation with five 500 ml. portions of distilled water. 1 N-NaOH (700 ml.) was then slowly added with stirring, the mixture stirred for 1 hr. and left overnight. Next, the resin was washed by decantation with five 2 l. portions of distilled water and, as a slurry in water, poured into the top of a column filled with water. It was washed with distilled water (backflow) until the *p*H of the effluent reached the range of 8.2 to 8.5. At this stage 0.2 M-sodium phosphate buffer of *p*H 6.0 ± 0.1, with thymol added as a preservative, was passed through the column until the effluent had a *p*H of 5.9 to 6.1. The resin was then kept in the sodium phosphate buffer until use. Amberlite IR4-B (analytical grade) was used to remove the hydrochloric acid in the eluate from the Amberlite IRC-50 columns.

Results

Stability.—There was a difference between the stability of extracts and urine. Two extracts were kept for 27 to 29 hr. at a temperature of 20 to 22° at various *p*H values between 0.3 and 13.5 and then tested for activity. Both extracts gave similar results; no loss was detected in the *p*H range 0.3 to 7.0. At *p*H 7.5 there was some loss and at *p*H 13.5 only 20% of the activity was left.

On the other hand, six urines were adjusted to *p*H 6, and kept at 20°; after 24 hr. only 50 to 70% of the activity was left and, in one specimen tested after 48 hr., only 12% was left. In this specimen kept at 4°, 75% of the activity remained after 48 hr.

Paper Pulp Chromatography.—Attempts to adsorb the active principle on paper pulp from aqueous solutions of extracts revealed that one active fraction passed quickly through the column. In addition, all the extracts analysed contained an active fraction that could not be eluted with water or with 0.1 N-acetic acid, but which was rapidly eluted with 0.2 N-HCl (Table I, Expt. 1). Experiments with increasing amounts of urine extract showed that the capacity of the columns for this active principle was 23 to 27 units/ml.

The urine extracts are buffers with *p*H values in distilled water ranging from 3.3 to 3.8. When the *p*H of the extract solutions was adjusted with sodium bicarbonate to about 6 prior to water chromatography most of the activity was lost (Table I, Expt. 2). When the column was first washed with 0.2 N-HCl and then freed of acid with distilled water, this loss was partially prevented (Table I, Expt. 3), possibly owing to the removal of impurities from the paper. In the method of purification described below the column was washed with 0.2 N-HCl and extracts contained 0.1 N-acetic acid (Table I, Expt. 4).

TABLE I

CHROMATOGRAPHY OF URINE EXTRACT ON PAPER PULP

The total activity in each experiment was 80 units. Column size 8 × 1 cm. (6.3 ml.). Flow 0.3 to 0.35 ml./min. In each experiment the column (a) gives the fraction numbers (each 20 ml.) and the column (b) gives the % recovery.

Expt.	1		2		3		4	
Column washed with	Water		Water		0·2 N-HCl		0·2 N-HCl	
Extract diluted with	Water		$NaHCO_3$		$NaHCO_3$		0·1 N-acetic acid	
*p*H of starting solution	3·8		6·0		6·0		2·9	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Eluted with water	1 2 4–10	65 2·5 0	1–6	0	1 2 3–6	13·5 9·0 0		
0·1 N-acetic acid	11–20	0	7–14	0	7–12	0	1 2 3–6	66·3 1·5 0
0·2 N-HCl	21 22 23–30	8·3 0·6 0	15 16 17–20	3·8 0 5 0	13 14 15–17	8·8 0·5 0	7 8–11	6·5 0
Total		76·4		4·3		31·8		74·3

Aluminium Oxide Chromatography.—Chromatography on aluminium oxide columns was carried out successfully by Andrade, Diniz, and Rocha e Silva (1953) for purification of bradykinin. Walaszek (1957) could recover no activity when using ordinary alkaline aluminium oxide columns for purification of the urinary constituents; but after washing out with hydrochloric acid, aluminium oxide columns yielded nearly 100% activity, provided the experimental conditions were otherwise favourable. In agreement with the experience of Walaszek (1957) we found that an alkaline aluminium oxide ("Woelm" basic) allowed recovery of very little active substance compared with an HCl-activated type ("Woelm" acid). An almost neutral aluminium oxide

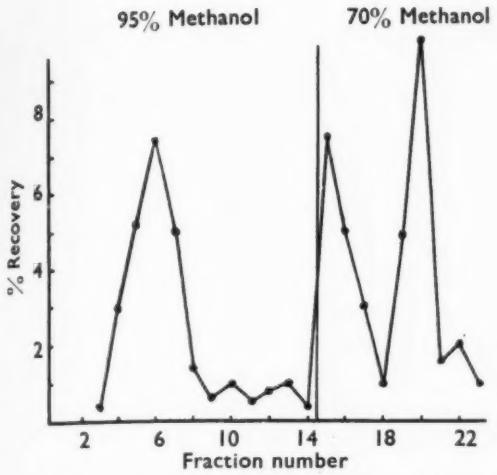

FIG. 1.—Chromatography of urine extract on aluminium oxide. Aluminium oxide (Woelm acid); column size, 135 mm. × 10 mm.; urine extract, 180 units; rate of elution, 0.08 to 0.10 ml./min.; fraction volume, 5 ml.; total recovery, 62.9%.

("Woelm" neutral) behaved like the alkaline type. Fig. 1 illustrates an experiment in which the active principle was adsorbed on aluminium oxide, "Woelm" acid, from 95% methanol, which was also used as the first eluent. When 13 fractions had been collected, the eluent was changed to 70% methanol. Most of the weight of the urine extract used passed into the fractions 2, 3, and 4, while these contained less than 5% of the original activity. Repeated experiments all showed one peak of activity in the 95% methanol fractions and two peaks of activity in the 70% methanol fractions, thus affording evidence that at least two active principles are present in the urine extract. The total activity recovered in the example in Fig. 1 was considerably higher than that found by Walaszek (1957) with an initial solvent of 95% methanol; it seems, however, to vary greatly with the size of the column. An experiment carried through with a 50% higher column and a 50% larger amount of urine extract, under otherwise equal conditions, gave total activity recovery of 32% against 63% in the above-mentioned experiment.

Ion Exchange Chromatography.—A method better suited for preparations of extracts than that of Gomes (1955), which was based on extraction of an acidified urine with an equal volume of *n*-butanol and precipitation with large volumes of ether, was desirable for isolation of the rat uterus-stimulating substances from urine. Ion exchange resins were tried as adsorbents for the active principles. The cation exchange resin Amberlite IRC-50 proved to be useful when pulverized to 40 to 60 mesh and buffered with sodium ions to *p*H about 6. The capacity was sufficient and the activity could be eluted with relatively small volumes of HCl. This resin was used by Andrade and Rocha e Silva (1956) for the purification of bradykinin.

In a typical experiment, the *p*H of a sample of urine containing 7.0 units/ml. was adjusted to 6 after which it was diluted with an equal volume of water and filtered. (The ion exchange column tended to clog if untreated urine was used, and dilution also increased the capacity of the resin.) The fluid was passed through an Amberlite IRC-50 column (50 by 5 mm.) at a rate of 0.30 to 0.35 ml./min. After 360 ml. (180 ml. of urine) had passed, the activity began to come out, and the adsorption was stopped. Fig. 2 shows how the active principles were eluted with 0.2 N-HCl. Fractions 1+2+3+4 contained in 20 ml. 85% of the eluted activity, and fractions 2+3 in 10 ml. 70%. The total recovery was, however, only about 60%. The shape of the elution curve in Fig. 2 suggested that the loss was unlikely to be due to incomplete elution. The loss may have been due to one or more substances having remained on the column or to destruction of activity on the column. Another possibility was that the activity had been lost in the urine prior to its passage through the column. The instability of untreated urine has already been described.

The anion exchange resin Amberlite IR4-B was found to be suitable for removing excess of HCl in the eluate from the Amberlite IRC-50 columns. 85 to 100% activity was usually recovered, and the passage of the active principles was not delayed by adsorption.

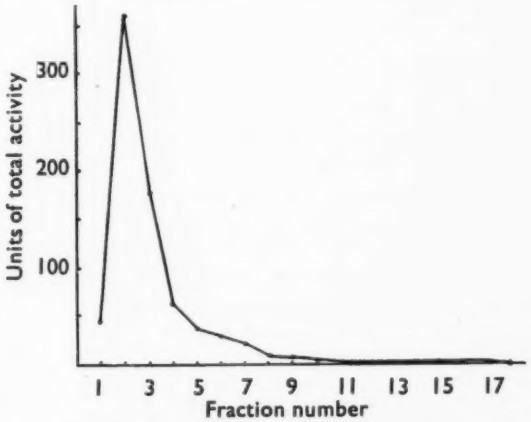

FIG. 2.—Elution of rat uterus-contracting substances from an Amberlite IRC-50 column. Elution fluid, 0.2 N-HCl; column size, 50 mm. × 5 mm.; rate of elution, 0.08 to 0.10 ml./min.; fraction volume, 5 ml.

Purification Procedure.—A typical example of purification procedure of the two rat uterus-stimulating principles in a 24 hr. urine specimen is described below.

To a 1,400 ml. sample of urine (5.3 units/ml.), NaOH was added to a *p*H of 6 and distilled water to produce a volume of 2,800 ml. After filtration, the fluid was passed through an Amberlite IRC-50 column (150 × 10 mm.) at a rate of about 2 ml./min. No activity could be detected in the urine which had passed through the column. The column was washed thoroughly with distilled water. Elution was performed with 0.2 N-HCl at a rate of 0.6 to 0.7 ml./min. and 300 ml. of eluate was collected (plus 300 ml. to control the effectiveness of the elution). The 300 ml. eluate was passed through an Amberlite IR4-B column (290 × 20 mm.) at a rate of 10 to 15 ml./min. This was followed by washing with distilled water to 375 ml. of eluate. The eluate contained 9.5 units/ml. and its *p*H was 8.6. Simple solids were removed by distributing this eluate in 5 dialysis casings ($\frac{4}{8}$ in.) in quantities of 75 ml. and dialysing against 1,200 ml. of distilled water for 3 hr. The outer fluid was changed four times at intervals of 15 min. and then four times at intervals of 30 min. The final *p*H of the inner fluid was 6.8. This process involved the loss of only about 15% of the total activity. Acetic acid to 0.1 M was added immediately after the dialysis.

The two active principles in a small amount of the dialysed acetic acid solution were then separated on a paper pulp column in order to assess the size of column required for the total quantity of active solution. A volume of 10 ml. (81 units) was passed through a column (80 × 10 mm.) at a rate of 0.3 to 0.4 ml./min. and the column was thereafter washed thoroughly with 0.1 N-acetic acid to remove the first active principle completely. Elution was then performed with 0.2 N-HCl and three 20 ml. fractions were collected for freeze-drying and assay. Only 4.3 units were present in the fractions collected, corresponding to 160 units in the total quantity of dialysate (375 ml.).

The amount of acetic acid dialysate left (365 ml.) was then passed through a paper pulp column (200 × 10 mm.) at a rate of about 1 ml./min. and 400 ml. of acetic acid eluate was collected. The volume of this column was about 15.5 ml., which should adsorb over 300 units of activity. The column was thereafter washed thoroughly with acetic acid, and eluted with 0.2 N-HCl. The acetic acid eluate (400 ml.) as well as the HCl acid eluate (110 ml.) were concentrated *in vacuo* at 25° and afterwards freeze-dried.

TABLE II

ISOLATION OF TWO RAT UTERUS-CONTRACTING FRACTIONS IN URINE

Recovery of activity at different stages of the isolation procedure (see text for further explanation of the table).

Fluids	Volume (ml.)	Activity		
		Units Total	%	Units/ml.
Urine	1,400	7,420	100	5·3
HCl eluate from Amberlite IRC-50	a: 300 (b: 300)	3,500 (720)	47 (10)	11.7 (2·4)
Eluate from Amberlite IR4-B	375	3,560	48	9·5
Eluate from Amberlite IR4-B after dialysis	375	3,038	41	8·1
Material not adsorbed on paper pulp	400	1,747	24	4·4
HCl eluate from paper pulp	110	161	2	1·4

Table II shows the activities recovered at the various stages of purification.

Comments on the Purification Procedure.—It is seen in Table II that two steps in the purification method cause a considerable loss of activity, namely the use of the Amberlite IRC-50 column and the adsorption on the paper pulp column. On the other hand, removal of HCl by means of Amberlite IR4-B and the dialysis caused only negligible losses. In the above experiment 47% of the activity was recovered from the Amberlite IRC-50 column (57% if the second eluate was included). In a number of similar experiments on different scales, the recovery varied between 35 and 70%. In these experiments the concentration of active substance in the HCl eluate varied between 10 and 25 units/ml.

The concentration of HCl was not very critical, but the amount of active principle eluted/ml. of HCl decreased if the HCl concentration was too high or too low. Under comparable conditions, eluate using 0.6 N-HCl was seen to contain 6 units/ml.; 0.2 N, 20 units/ml.; 0.1 N, 22 units/ml.; and 0.01 N, only 4 units/ml. In large-scale experiments, it was found expedient to start the elution with a stronger HCl, such as 1 N, to reduce as much as possible the volume required to elute the activity. As soon as the buffer effect of the column had been broken, the concentrated HCl was replaced with 0.2 N-HCl.

While the recovery from Amberlite IRC-50 varied appreciably in different experiments, that involved in separation of the two active fractions on paper pulp remained fairly constantly at 60 to 70%. The principle adsorbed and eluted with HCl generally constituted a small proportion from 2 to 10% of the recovered activity.

The residues after freeze-drying were very hygroscopic. The most active material so prepared contained 100 to 120 units/mg. but proved to be very unstable.

Discussion

The method described for adsorption of the rat uterus-stimulating principles in human urine on the cation exchange resin Amberlite IRC-50 seems to constitute a useful basis for isolation of these principles, though alterations that led to increased recovery from the column would favour its use. Very active fractions were obtained from the extracts by adsorption on paper pulp followed by elution with acid.

The experiments with aluminium oxide (Fig. 1) and with paper pulp (Table I) confirmed the conclusion of Walaszek (1957) that human urine contains more than one substance causing contraction of the rat uterus.

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THE MODE OF ACTION OF QUINIDINE ON ISOLATED RABBIT ATRIA INTERPRETED FROM INTRACELLULAR POTENTIAL RECORDS

BY

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An attempt has been made to show why quinidine, which has long been known not to lengthen the duration of the cardiac action potential, measured with external electrodes, and also not to lengthen, and sometimes to shorten, the absolute refractory period, nevertheless reduces the maximum frequency at which atria can respond to a stimulus. Simultaneous measurements have been made in electrically driven isolated rabbit atria of contractions, conduction velocity and intracellular potentials before and during exposure to a wide range of concentrations of quinidine sulphate. The resting potential remained undiminished, in contrast to the effect of quinidine on Purkinje fibres. In the therapeutic range of doses, up to 10 mg./l., the half-time for repolarization was either shortened or unchanged, thus providing an explanation for the failure of quinidine to prolong the absolute refractory period. In contrast, even at low concentrations of quinidine, conduction velocity and the rate of rise of the action potential were greatly slowed, and the height of the overshoot was reduced. The terminal phase of the action potential was prolonged. It is known that the rate of rise of the action potential is a function of the level of repolarization at which an impulse takes off (the more negative the take-off point, the faster the rate of rise). Normally, a stimulus introduced when repolarization has proceeded to 2/3 of the resting potential evokes a response with a rate of rise fast enough for propagation, so that the duration of the terminal 1/3 of the phase of repolarization has no influence upon the length of the effective refractory period. In the presence of quinidine, however, the rate of rise itself was directly reduced, thus repolarization had to proceed further before the critical take-off point was reached at which the rate of rise was fast enough for propagation, and the duration of the terminal phase of repolarization thus became significant. It has been concluded that quinidine prolongs the effective refractory period by slowing the phase of depolarization, without any change necessarily occurring in the half-time for repolarization, which determines the absolute refractory period. Acetylcholine accelerated the rate of rise of the action potential even in the presence of high concentrations of quinidine.

The importance attached to the various actions of quinidine has to some extent been affected by views about the nature of fibrillation. Lewis, Drury, Iliescu, and Wedd (1921) originally claimed that the absolute refractory period of the atrium of the dog was prolonged by quinidine, and regarded this as evidence in support of the hypothesis that fibrillation consisted of a circular sequence of excitation. When it was shown subsequently by Love (1926) that quinidine did not, in fact, prolong the absolute refractory period, but sometimes shortened it, Lewis and Drury (1926) nevertheless retained the "circus" hypothesis, on the ground that quinidine prolonged what they called the "effective" refractory period; that is, quinidine prolonged the minimal interval at which *propagated* responses could be elicited by stimulation. The evidence relevant to the various hypotheses concerning the nature of fibrillation has been extensively reviewed (Dipalma and Schults, 1950; Prinzmetal, Corday, Brill, Oblath, and Kruger, 1952).

There is no doubt that quinidine reduces the maximum frequency at which atria can respond to stimulation, and the prolongation of the effective refractory period by quinidine has been confirmed by several authors (Dawes, 1946; Gold, 1950; Dawes and Vane, 1956). The paradox remains that quinidine does not prolong the absolute refractory period, that is the shortest

interval after a first stimulus at which a local response can be elicited by a second stimulus. The absolute refractory period of cardiac muscle has long been known to be approximately equal to the duration of the action potential (Adrian, 1921), and the latter also was shown not to be prolonged by quinidine (Wedd, Blair, and Gosselin, 1942). More precise information concerning the action of drugs on the electrical activity of cardiac muscle can now be obtained by means of intracellular electrodes. Studies of the effects of quinidine on intracellular potentials have been made in Purkinje fibres by Weidmann (1955b) and Corabœuf, Boistel, and Distel (1956), and in guinea-pig ventricle by Johnson (1956). The differences between atrial muscle, and ventricular muscle and Purkinje tissue, are considerable. Neither Purkinje tissue nor mammalian ventricle is sensitive to acetylcholine (ACh), and the antagonism between quinidine and ACh has been the subject of much investigation (Dawes, 1946; de Elio, 1948; Rand and Walker, 1958). As the resting potentials and the shapes of the intracellular action potentials are dissimilar, the action of a drug on ventricle or Purkinje tissue cannot be assumed to be the same on atrial tissue. Since antifibrillatory drugs are employed clinically for their effects on atrial muscle, it was thought worth while to investigate the action of quinidine on atria.

Weidmann (1955b) examined the effects of quinidine at one concentration only, 10 mg./l., and in association with a slowing of depolarization there was a large fall in resting potential, from −98 mV. to −72 mV. No such fall has been observed in the present experiments. Corabœuf *et al.* (1956) also examined the effect of quinidine at one concentration only, 100 mg./l., a level which would probably be lethal in man. These authors likewise observed a fall in resting potential, and a flattening of the plateau of the action potential. Since there is no plateau in the atrial action potential, it was clear that the effect of quinidine on atrial muscle itself required investigation.

Clinically the dose required to arrest fibrillation is very critical (Gold, 1950) and the actual serum concentration in 75% of a series of successfully treated patients was found to lie between 4 and 9 mg./l. with a mean of 5.9 mg./l. (Sokolow and Edgar, 1950). It is difficult to estimate the extent to which concentrations bathing an isolated tissue can be regarded as equivalent to therapeutic or toxic serum levels in man. For this reason, in the present experiments measurements of contractions have been made simultaneously with the intracellular records, in order that the toxicity of the various concentrations of quinidine could be independently assessed in relation to their effects on electrical activity.

Methods

The apparatus has already been described (Vaughan Williams, 1955, 1958). Rabbit atria were placed horizontally in a bath whose temperature was controlled at 31° to within ±0.1°. Contractions were recorded with an RCA 5734 transducer, and displayed on one beam of a Dumont 322 oscilloscope. Intracellular potentials were displayed on the other beam. External potentials, from which conduction velocity was calculated, were recorded from right and left atria with pairs of small platinum bipolar electrodes, and were displayed on a Cossor 1049 oscilloscope. The screens of both oscilloscopes were photographed simultaneously with a Grass camera; the time at which each exposure was made was also recorded to within 0.1 sec. The atria were driven electrically through Ag-AgCl electrodes placed on the tip of the left atrium. All intracellular records were taken from the endocardial surface of the right atrium, some distance from the natural pacemaker. The oscilloscope sweep was started by the stimulus, and at the end of its traverse was itself made to produce a pulse which set in motion the mechanism for shifting one frame in the camera; this in turn operated a shutter to record the time. All records were thus obtained on stationary film, the frames being changed automatically between each heart beat. The concentrations of quinidine have been expressed as w/v of the sulphate.

The observations photographed on the film were subsequently measured on the lined screen of a specially made projector. The rate of rise of the action potential was calculated from direct measurements of the angle of its slope. This method was laborious but permitted the measurement of differences of 3% in the rate of rise, and was preferred to measurement by electrical differentiation which involved greater difficulties in calibration and measurement. The rates of stimulation selected were 10% higher than the natural frequency of the spontaneously beating preparations. The natural pacemaker frequencies had a range of 90 to 160 beats/min.

Results

Effects of Non-toxic Concentrations

Simultaneous records of the contractions, conduction velocity and intracellular potentials of isolated rabbit atria were made in 29 experiments before and during the action of various concentrations of quinidine ranging from 10^{-7} to 6×10^{-5}. Quinidine at a concentration of 10^{-7}

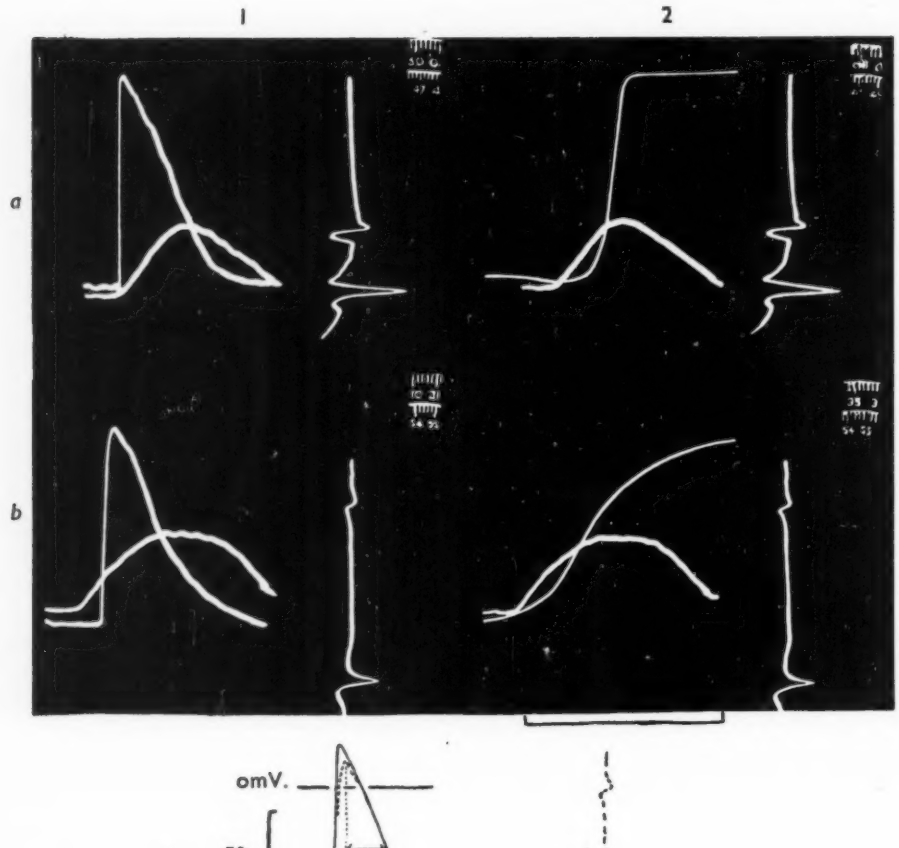

FIG. 1.—The effect of quinidine on contraction, conduction velocity and intracellular potential. *a*, Controls. Left-hand frame (1). The upper trace is the intracellular potential at a slow sweep speed. Immediately below it is the record of contraction at the same sweep speed. The vertical trace to right records external action potentials from left and right atria, from which conduction velocity can be calculated. The figures in the inset show the time at which the photograph was taken to within 0.1 sec. (see text). Right-hand frame (2). The trace recording intracellular potential has been speeded up about × 40, so that only the upstroke of the action potential is seen. *b*, Records from the same area after one hour of exposure to quinidine 9×10^{-6}. Conduction velocity and rate of rise greatly reduced. The time scale (5 msec.) for the fast sweep in *a*2 and *b*2 is given below *b*2. *c*, Tracings of *a*1 and *b*1 superimposed, with time and voltage scales. The height of the action potential was reduced by quinidine, but the duration was unchanged, except at the tail. The duration of 1/2 repolarization (R) was actually reduced.

had no effect upon any of the activities measured. In the presence of quinidine 6×10^{-5}, the atria became electrically inexcitable, and so attention has been directed to the effects of concentrations between these extremes. In order that the atria should follow the stimulus rather than their natural pacemaker, the frequency of stimulation had to be a little faster than that of the pacemaker. Stimuli of 5 msec. duration were employed. The stimulus strength was gradually increased until the atria began to follow, and was then doubled. In the presence of concentrations of quinidine 10^{-5} or less the atria were always still able to follow the stimulus at the control frequency, although the strength and duration of the stimulus had sometimes to be increased, by not more than 50%.

The effect of quinidine 9×10^{-6} is shown in Fig. 1. The top left-hand frame shows the intracellular potential at a slow sweep speed and immediately below it a record of the contraction at the same sweep speed. The external potentials have been displayed on the vertical trace to the right of this, and in the top right-hand corner the inset indicates that the exposure was taken at 47 min. (lower number) 0.3 sec. The top right-hand frame was taken a few seconds later, at 47 min. 6.1 sec., the speed of the oscilloscope sweep recording the intracellular potential having meanwhile been increased about 40 times. Thus only the upstroke of the intracellular potential can be seen on this trace. The two frames formed part of a series of control observations whose results have been summarized in the line relating to

experiment 15 in Table I. When the set of control observations was complete the atria were exposed to quinidine 9×10^{-6}, and after an hour a second series of observations was made, from which the lower two frames in Fig. 1 have been taken.

A major difficulty in making comparisons of this kind was the variation in the records of intracellular potentials taken from different sites. The rate of rise and the duration of the action potential were found to be more variable than the resting potential and the height of the action potential. The fibres were far too small to be seen individually, and it was, therefore, impossible to return to the same fibre for a second observation. Fortunately, however, fibres in the same bundle usually gave very similar records, although they might differ from those in a neighbouring bundle. A map of the bundles in the microscope field was therefore drawn and three or four sites were numbered. Several entries were made at each site and the records were compared with others taken subsequently from the same site during exposure to quinidine. The lower two frames in Fig. 1(*b*) were thus records taken 1 hr. 7.5 min. later from the same site as that from which the upper records were taken. Comparison of the two right-hand frames indicates that conduction velocity and the rate of rise of the action potential had greatly decreased in the presence of quinidine. Tracings of the records from the left-hand two frames have been superimposed in Fig. 1(*c*), and show that the height of the action potential had been decreased by quinidine. The resting potential (measured from a zero line on a succeeding frame, not shown) had actually increased by 3 mV., although there was no statistically significant change in resting potential calculated from all the observations in this experiment. The main change was a large reduction in the "overshoot." The duration of the action potential measured at half the action potential height was unchanged, although the tail of the repolarization phase was a little prolonged. Since, however, the phase of depolarization was greatly lengthened, the phase of repolarization alone, measured as the time from the peak of the action potential to half-repolarization (the distance R in Fig. 1), was actually shorter in the presence of quinidine. Thus quinidine, so far from slowing down the flow of ionic current responsible for repolarization, may actually speed it up as far as the half-repolarization point. Hoffman, Kao, and Suckling (1957) showed that a local response could occur in cardiac muscle when repolarization had reached only half the original resting potential. The absolute refractory period was approximately equal to the time taken to repolarize to half the resting value. An acceleration of the first half of the repolarization phase would thus explain why quinidine, in spite of slowing down the depolarization phase, does not prolong and may even shorten the absolute refractory period (Love, 1926).

Effects of Concentrations Above 16 mg./l.

When the concentration of quinidine was raised to 1.6×10^{-5}, the atria no longer followed a stimulus at the control frequency. In order to continue driving the atria, the frequency of stimulation had to be reduced, usually by only a few beats/min., but in the presence of the highest concentrations of quinidine, by as much as half the control rate. As soon as the rate was lowered the observations could no longer legitimately be compared with the control records. In normal atria merely reducing the frequency of stimulation prolonged the duration and increased the rate of rise of the action potential, and such effects were exaggerated in the presence of quinidine. No control observations at the reduced frequency necessitated by high quinidine concentrations could be obtained in normal atria, because the latter followed their natural pacemaker instead of the stimulus as soon as the frequency of stimulation was reduced. If the pacemaker region was cut away the value of the measurements was made uncertain by ignorance of the extent to which trauma might have interfered with normal activity.

The upstroke of the action potential may be divided into three phases: (1) a slow "foot," (2) a fast and almost linearly rising central portion, (3) a more slowly rising terminal phase. Although quinidine caused a slowing of the rate of rise of the central portion as well, it became clear that the terminal phase exhibited the greatest slowing, as is shown in Fig. 2. The effects of increasing concentrations of quinidine are shown in four experiments. In *a* the control observations have been given as well as those illustrating the effect of quinidine 5×10^{-6}. In *b*, *c*, and *d* records taken in the presence of quinidine only are shown. In *d*, the presence of quinidine 6×10^{-5}, the atria eventually became inexcitable, but the records indicate that conducted electrical responses continued to occur after the contractions had ceased to be measurable, since the intracellular electrode was in the right atrium but the stimulus was applied to the left atrium.

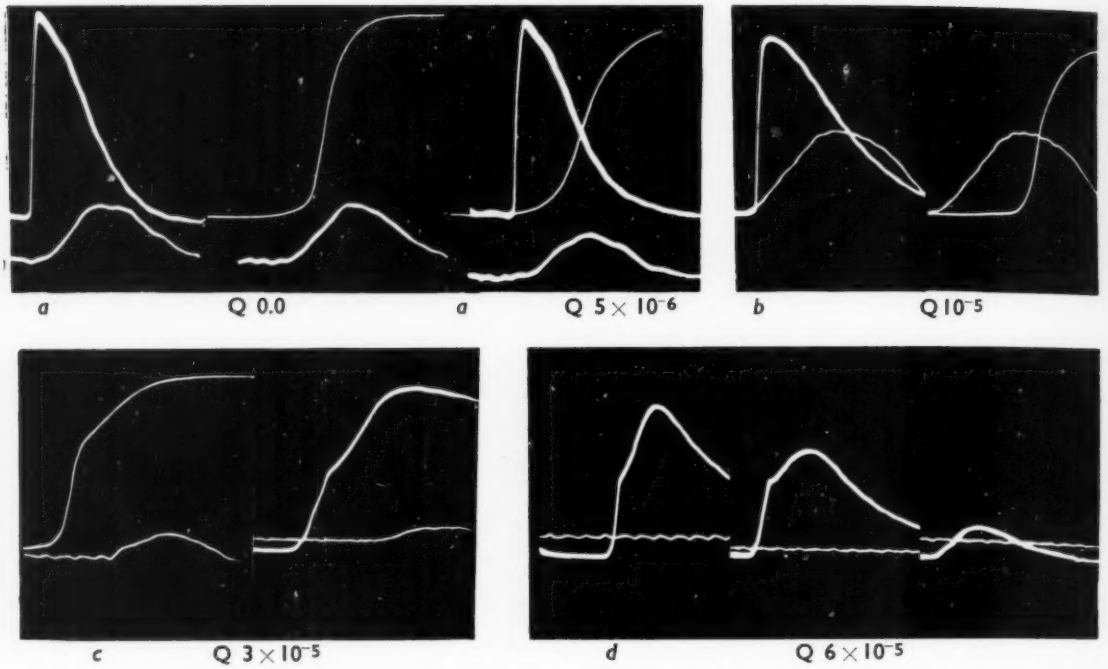

FIG. 2.—*a* to *d*. Effect of increasing concentrations of quinidine (Q) on contraction and intracellular potential. It is clear that the terminal part of the upstroke of the action potential was especially retarded. In *c* and *d* the traces were all at the slow sweep speed. In *d* contractions were no longer measurable although the action potentials were conducted.

When the atria began to fail in the presence of high concentrations of quinidine, they would sometimes do so by responding, say, to three stimuli, but failing to respond to the fourth. When the fifth stimulus arrived, after the interval of the missed beat, the atrium was found to have recovered sufficiently during its period of rest to respond with a larger contraction, and a faster rate of rise of the action potential. Conduction velocity was also increased. In Fig. 3*a*, an action potential at the beginning of failure in the presence of quinidine 3×10^{-5} is shown, with a third phase of depolarization so slow that a concavity had been formed in the upstroke. The next record depicts the responses to six successive stimuli photographed on the same frame. The first occurred just after a double interval, so that the contraction was large and there was no concavity in the upstroke of the action potential. In the responses to the next three stimuli the concavity or "step" became more and more pronounced, and the contractions progressively smaller. The atria failed to respond to the fifth stimulus altogether, but during the interval of the missed beat recovery again took place to such an extent that the sixth response resembled the first. The resting potential was unchanged throughout.

Effects of ACh in the Presence of Quinidine

Briscoe and Burn (1954) showed that when isolated rabbit atria had stopped in high concentrations of quinidine, they could be restarted with ACh. Marshall and Vaughan Williams (1956) found that atria stopped by cooling could also be restarted by ACh, and it was suggested that both phenomena might be explained by the effect of ACh in increasing the rate of entry of depolarizing ions (Marshall, 1957; Vaughan Williams, 1958). The present experiments provided the opportunity of observing directly the effect of ACh on the rate of depolarization in the presence of quinidine, and a measurement of this kind is shown in Fig. 3*b*. After control observations had been made, examples of which are illustrated, the atria were exposed to quinidine 2.5×10^{-6}, whereupon they failed to follow the stimulus at the original frequency. The frequency was then lowered (from 120 to 105 stimuli/min.) until the atria again followed every stimulus, but the reduction in frequency resulted in larger contractions and a greatly prolonged duration of the phase of repolarization. There was, nevertheless, still a marked concavity in the upstroke of the action potential. ACh was then added to the bath to give a final concentration of 10^{-6} (w/v), and the

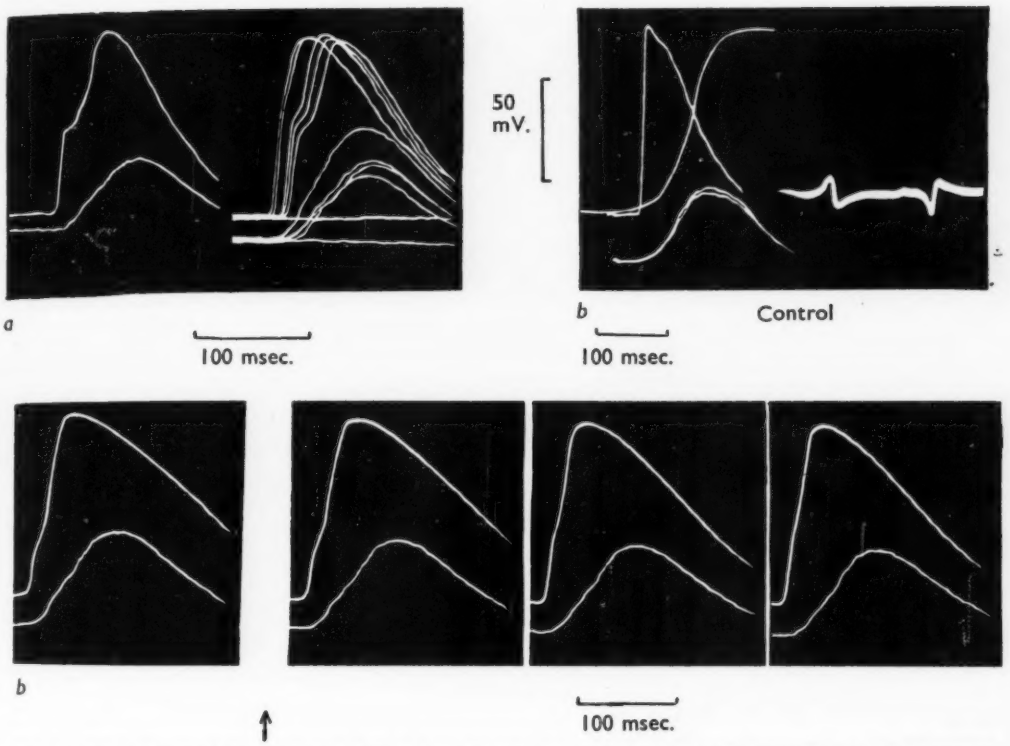

FIG. 3.—Effect of lowered frequency and ACh in increasing the rate of rise of the action potential. *a*. In the presence of quinidine 3×10^{-5}. Left, atria still responding to every stimulus. Right, responses to six successive stimuli. The first response shown followed a missed beat, so that the rate of rise was faster, without a "step," and the contraction was larger. The step appeared progressively in the upstroke of the action potential during the next three beats and contractions became smaller. The response failed altogether on the fifth stimulus, so that the response to the sixth, after the interval of the missed beat, resembled that to the first. *b*. Experiment to illustrate the effect of ACh 10^{-6}. At bottom left the frequency was reduced until responses occurred to every stimulus in the presence of quinidine 25 mg./l. There was nevertheless a "step" still apparent, which was almost abolished when ACh speeded up the rate of rise of the action potential. The lower records were all made in the presence of quinidine 2.5×10^{-5}. At the arrow, ACh 10^{-6} was added to the fluid bathing the preparation.

succeeding photographs show that it caused an increase in the rate of rise of the action potential, especially in the third phase, so that the concavity almost disappeared.

Analysis of Results

The effects of quinidine on contractions, on the intracellular resting and action potentials, and on the duration of the first half of the phase of repolarization in all the experiments have been summarized in Table I. Each estimate represents the mean of several sets of observations taken at different sites in each experiment, and the standard errors of the means have been indicated when there were four or more sets of observations. The horizontal broken line divides those experiments in which the atria still followed the stimulus at the control frequency from those in which the frequency had to be reduced It is evident that quinidine, except in one experiment, had no dramatic effect on the resting potential, but caused a highly significant ($P<.001$) reduction in the height of the overshoot. It has been concluded that the action of quinidine cannot be attributed to a change in resting potential. This contrasts with the effects of quinidine on Purkinje tissue (Weidmann, 1955b).

The duration of the first half of the phase of repolarization was either shortened or not significantly ($P>.05$) changed in the presence of quinidine 10 mg./l. or less. Above 16 mg./l. it was necessary to reduce the frequency of stimulation, and there is evidence (Hoffmann and Suckling, 1954) that this alone could lead to a prolongation of the duration of the action potential. It was not possible to say, therefore, to what extent the lengthening of the phase of depolarization in the presence of toxic concentrations of quinidine was due to the quinidine or to the reduction in frequency. It may be noted, however (Fig.

TABLE I

EFFECTS OF QUINIDINE ON CONTRACTION AND ON INTRACELLULAR RESTING AND ACTION POTENTIALS

The results given above the horizontal broken line are those in which the atria followed the stimulus at the control frequency, while in those below the frequency had to be reduced.

Expt. No.	Conc.	% Change in Contraction	Resting Potential (mV.)		Action Potential (mV.)		Time for Half Repolarization (msec.)	
			Before	During	Before	During	Before	During
2	10^{-6}	0	72 ±0·6	78 ±4	98 ±0·6	101 ±0·4	58 ±0·3	57·5±0·6
3	$2{\cdot}5\times10^{-6}$	−26	69 ±0·4	68·6±0·7	87 ±0·4	87·2±0·1	59	58
4	3×10^{-6}	−11	72 ±0·6	72·3±1·2	98 ±0·7	92·5±0·8	58 ±0·3	56·6±0·9
5	5×10^{-6}	−20	74·5±0·7	74·3±0·5	102 ±0·5	92 ±0·1	85 ±1·7	74 ±0·3
6	,,	−26	73·5±1·3	70 ±0·3	93 ±0·7	81·5±0·5	51 ±0·4	36 ±3·4
7	,,	−2	72·3±0·3	71·6±0·4	94·5±0·2	88·5±0·5	60·7	62
8	,,	0	73·2±0·2	72 ±0·2	96 ±0·1	90·2±0·2	53·2±3	51·8±0·4
9	,,	−2	73·2±0·2	74 ±0·4	99 ±0·2	93·5±0·1	58 ±0·6	57·3±0·2
10	6×10^{-6}	−7	71·5±0·5	72·5±1·3	84·5±0·2	85 ±0·7	41 ±0·4	42 ±0·1
11	7×10^{-6}	−27	74·2±0·7	77·4±1·2	99·1±0·9	95·5±1·0	61·7±3·8	61·5±2·7
12	8×10^{-6}	−1·5	73 ±0·3	76 ±0·5	95 ±0·3	93·5±0·7	62·5±0·6	69 ±0·4
13	,,	−4	71 ±1·9	70 ±0·9	99·5±2·1	90 ±1·1	70·5±1·2	87 ±2
14	,,	−3	77·8±1·4	73 ±1·6	98·5±0·8	90 ±0·7	57·7±1·4	56·8±2
15	9×10^{-6}	+6	80·5±1·3	82·5±2·2	102 ±2·5	94 ±1·7	64·3±6	53·1±3·8
16	10^{-5}	−25	76	77	97·5	93·5	39	44·5
17	,,	−21	72·3±0·3	71·3±1·2	94·5±0·2	87 ±0·7	61	70
18	,,	−24	73·2±0·2	69·5±0·4	96 ±0·1	88·7±0·2	53·2±3	58·5±0·3
19	,,	−32	73·2±0·2	71·3±0·6	99 ±0·2	92·6±0·2	58·6±0·5	67·5±0·9
20	$1{\cdot}6\times10^{-5}$	−17	71 ±1·6	70·5±1·3	99·5±2·0	86 ±0·7	71 ±0·9	92 ±3
21	,,	−17·5	77·6±1·4	68·5	98·5±0·8	81 ±0·9	58 ±1·2	92
22	$2{\cdot}5\times10^{-5}$	−52	72 ±0·8	72 ±0·6	93·5±1·2	86 →72	72	122
23	,,	−66	72 ±0·6	< 49	98 ±0·6	< 42	58 ±0·4	>61
24	$2{\cdot}6\times10^{-5}$	−29	71 ±1·7	72·5	99·5±2·1	80 ±0·9	70 ±1·6	>92
25	3×10^{-5}	−57	72·3±2·7	68·3±0·4	94·5±0·2	< 89	61 ±2	65·4±2·6
26	,,	−63	73·2±0·2	67·7±0·3	96 ±0·2	< 87	53 ±3	55·5±6
27	,,	−49	73·2±0·2	71	99 ±0·1	84 →73	58	>69
28	4×10^{-5}	−31	71 ±1·5	71·2	99·5±1·7	84 →61	70	146

6), that in three experiments in concentrations above 16 mg./l. the prolongation was not great in spite of the quinidine *and* the reduced frequency. It is apparent that a change in the first half of the phase of repolarization cannot be concerned in the mode of action of therapeutic levels of quinidine. The absence of change, or the actual shortening of this phase in some experiments, is of interest, however, since it provides an explanation for the failure of quinidine to prolong the absolute refractory period.

The variation between atria was wide, and the graphs shown in Figs. 4, 5 and 6 have been drawn in order that comparisons may be made between experiments without the complicating factor of different control values for each experiment. The means of the control values of all the observations of action potential (98 mV.), resting potential (73 mV.) and half-time of repolarization (56 msec.) have been drawn as horizontal lines in Figs. 4, 5 and 6 respectively. The changes produced by quinidine in each experiment have then been plotted as changes from the common mean control in proportion to the actual change observed from each individual control. In this way the trends of the changes produced by increasing concentrations of quinidine have been clearly brought out. The vertical lines separate observations taken at this control frequency from those taken at a reduced frequency. There was no evidence of a change in resting potential in the presence of quinidine 10^{-5} or less. At higher concentrations there was a slight downward trend, but it was not highly significant ($P<.03$). In contrast, there was a striking and significant fall in the height of the action potential ($P<.001$) which would have been larger at concentrations of quinidine greater than 10^{-5} had it not been necessary to reduce the frequency of stimulation at these high concentrations.

Conduction velocity was always reduced by quinidine, and the change was doubtless a direct consequence of the slower rate of rise of the action potential. The maximum rate of rise was measured from the tangent of the fast central portion of the upstroke. The mean rate of rise was calculated from a measurement of the horizontal distance between the points at which the rising line of the upstroke cut horizontal lines 2.5 mV. above the most negative and 2.5 mV. below the most positive values of the action potential (*vide* Vaughan Williams, 1958). The measurements of maximum and mean rates of rise of the action potential and of conduction velocity are given in Table II, and the changes in the maximum and mean rates of rise produced by quinidine, referred to the mean of all the controls, have been plotted in Fig. 7.

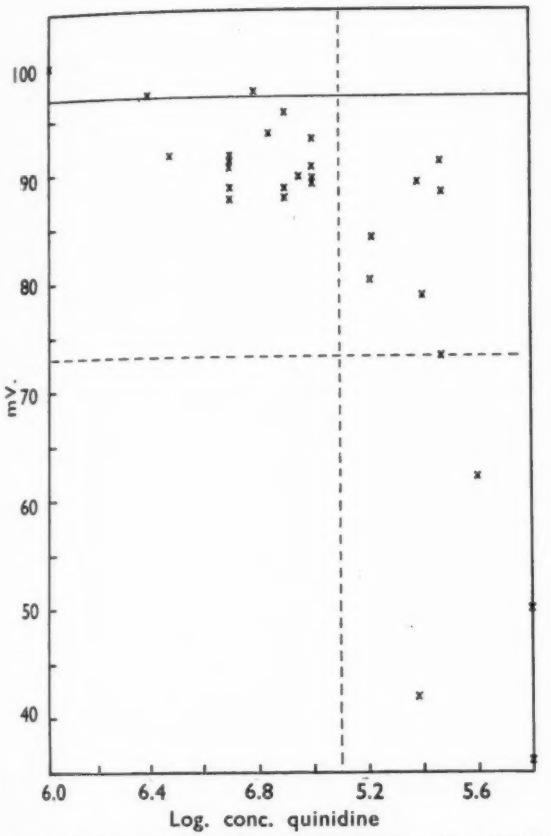

FIG. 4.—Effect of quinidine on the height of the action potential. Ordinate: millivolts. Abscissa: Log concentration of quinidine. The horizontal solid line represents the mean of all the control action potential heights. The horizontal broken line represents the mean of all the control resting potentials. The vertical broken line divides results taken from atria following the stimulus at the control frequency from those taken at a reduced frequency on the right.

The Effect of Quinidine on Contractions

Quinidine always produced a relaxation of the resting length of the atria. Provided the concentration was not greater than 10^{-5}, the effects on contraction were small (Table I). It is clear from Fig. 1 that in this experiment, although the contraction was no smaller in the presence of quinidine than in the control, its peak occurred later. The time from the beginning of the contraction to its peak was measured in all experiments, but although quinidine usually increased this duration by a few msec. the variation in the zero-peak time in control contractions was such that the change produced by quinidine was not statistically significant ($P>.05$).

Results During Recovery

In six experiments (Nos. 8, 11, 13, 15, 18 and 19) observations were made at intervals for more than an hour after the atria had been returned to the control solution from the quinidine solution. The changes produced by quinidine were slowly reversed, but only in experiment 8 (after quinidine 5×10^{-6}) was recovery complete at the end of one hour. A puzzling observation, made in all the experiments, was that during the early phase of recovery the duration of the action potential became *longer*. In the experiments 11, 13, 15, 18, and 19 the durations of the first half of repolarization became 71.4, 94, 80.4, 68 and 81 msec. respectively, which may be compared with the observations made before and during exposure to quinidine given in Table I. No explanation is offered for this phenomenon, but it is mentioned because the effect was regularly observed.

DISCUSSION

A puzzling feature of the action of quinidine has been that while there was no doubt that the maximum frequency at which atrial muscle could

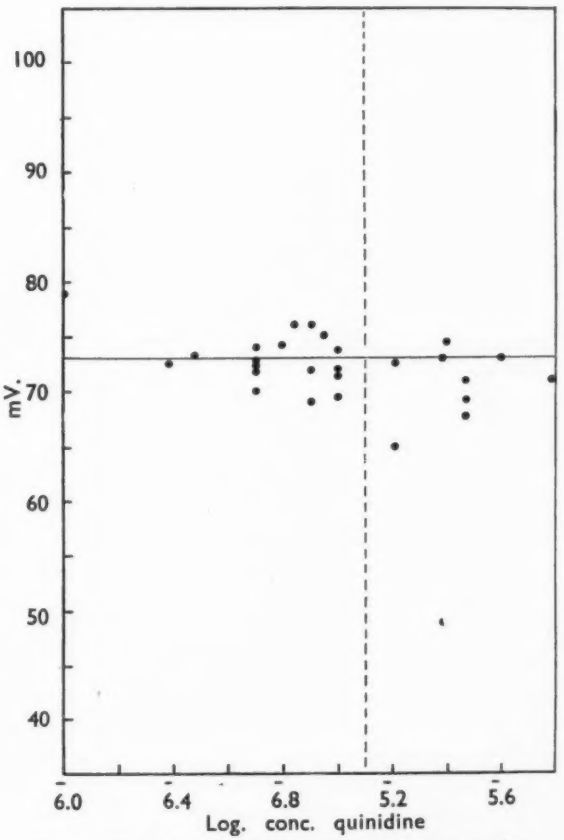

FIG. 5.—Effect of quinidine on the resting potential. Ordinate: millivolts. Abscissa: log concentration. The horizontal solid line represents the mean of all the control resting potentials. For explanation of vertical broken line, see legend to Fig. 4.

TABLE II

EFFECTS OF QUINIDINE ON CONDUCTION VELOCITY AND ON THE MEAN AND MAXIMUM RATES OF RISE OF THE INTRACELLULAR ACTION POTENTIAL

See Table I for explanation of dotted line.

Expt. No.	Conc.	Maximum Rate (V./sec.)		Mean Rate (V./sec.)		Conduction Velocity (M./sec.)	
		Before	During	Before	During	Before	During
2	10^{-6}	64 ±3	64 ±4	18·8	18·4	0·46	0·454
3	$2{\cdot}5\times10^{-6}$	47·4±1·3	22·1±0·3	14·2±0·3	7·5±0·2	0·634	0·300
4	3×10^{-6}	64 ±5	34·4±5·1	19·6	12·4	0·464	0·420
5	5×10^{-6}	107 ±1·2	59 ±4	20·2	12·6±0·4	0·308	0·185
6	,,	48 ±4	22 ±2	16·5±0·2	10 ±0·5	0·306	0·245
7	,,	56 ±3	20·3±0·8	25 ±0·4	12 ±0·2	0·368	0·327
8	,,	52·3±1·4	32·8±1·2	31 ±0·3	16·3±0·9	0·391	0·333
9	,,	45·6±3·2	37·5±1·8	28·1±0·8	15·3±0·3	0·388	0·340
10	6×10^{-6}	75 ±11	57 ±4·5	17·6±0·5	15·1±0·2	0·662	0·565
11	7×10^{-6}	90·5±5	58 ±6·5	29 ±2·1	12·2±2·2	0·332	0·235
12	8×10^{-6}	78·3±1·6	60·2±2·4	30 ±1·2	19·9±0·7	0·444	0·388
13	,,	83 ±2·1	41·5	34·2±1·3	12·9	0·441	0·375
14	,,	64 ±5	46 ±7	20·8±0·9	19·3±0·3	0·410	0·386
15	9×10^{-6}	120 ±9·2	36 ±4·8	54 ±12	16 ±1·4	0·720	0·270
16	10^{-5}	65·5	48			0·710	0·530
17	,,	56 ±3·2	16·6±3	25 ±0·36	7·7±2·1	0·368	0·246
18	,,	52·3±1·4	28·8	31 ±0·3	10·5±1·1	0·391	0·232
19	,,	45·9±3·7	25·7±4·8	28·1±0·8	9·7±0·6	0·386	0·246
20	$1{\cdot}6\times10^{-5}$	83 ±2·9	21 ±1·7	34 ±2	7·8±1·2	0·440	0·298
21	,,	64 ±5·1	33 ±3	21 ±1·2	9·1±0·4	0·420	0·247
22	$2{\cdot}5\times10^{-5}$	55	3·4	17·2	2·1	0·470	0·360
23	,,	64 ±4	< 7·3	19·6	4·2	0·460	0·280
24	$2{\cdot}6\times10^{-5}$	83 ±3	< 14·9	34 ±1·6	3·9±0·6	0·440	0·242
25	3×10^{-5}	56 ±3	< 7·15	25·2±0·4	< 2·3	0·368	0·240
26	,,	52·3±1·4	< 9·6	31 ±0·3	< 3·1	0·390	0·210
27	,,	45·6±3·2	< 7·6	28·2±0·7	< 2·6	0·380	0·222
28	4×10^{-5}	83 ±2·1	< 7·4	34·6±1·1	< 2·8	0·440	0·204

respond to a stimulus was reduced, that is, the "effective" refractory period was lengthened, there was also evidence that the duration of the action potential, measured with external electrodes, was not prolonged by quinidine (Wedd *et al.*, 1942), and that the absolute refractory period, defined as the minimum interval at which a *local* non-propagated response could be elicited, was unchanged or shortened (Love, 1926). In the present experiments an attempt has been made to resolve this paradox by an analysis of intracellular action potentials recorded from single fibres of isolated rabbit atria in the presence of a wide range of concentrations of quinidine.

The Importance of Concentration

Simultaneous measurements have been made of contraction, conduction velocity and intracellular potential in isolated rabbit auricles in the presence of a wide range of concentrations of quinidine sulphate. A major difficulty in interpreting the results has been to decide what concentrations in the isolated organ bath correspond to therapeutic and toxic levels of the drug in man. Sokolow and Edgar (1950) measured the concentrations of quinidine in the blood of thirty patients in whom auricular fibrillation had been successfully terminated. The average serum concentration was 5.9 mg./l., and 75% of the results fell between 4 and 9 mg./l. In some patients the concentrations reached were sufficient to evoke toxic manifestations. Nausea was experienced by several patients whose serum levels were 2, 5, 6.3, 7.0 and 10 mg./l. Tinnitus occurred in patients at 2.2, 4.3, 4.6, 5.4, and 11 mg./l.; diarrhoea at 4.2 and 6.3 mg./l.

In the present experiments an independent estimate of toxicity could be made from changes in the contractions which were simultaneously recorded. At concentrations of 10 mg./l. or less the contractions were not reduced by more than a quarter, and the atria were always able to follow the stimulus at the control frequency which was a little faster than the original frequency of their natural pacemaker. At concentrations of 16 mg./l. and above the reductions in the force of contraction became much larger, and the atria would no longer follow the stimulus, so that the frequency of stimulation had to be reduced.

At concentrations of quinidine of 10 mg./l. and less the results were quite clear cut. Quinidine produced no significant change in resting potential, nor was there any lengthening of the time required for repolarization to half the height of the action potential. There was, however, a prolongation of the tail of the repolarization phase. In all the experiments, at concentrations between 1.0 and 10 mg./l. inclusive, there was a great slowing in the rate of rise of the action potential,

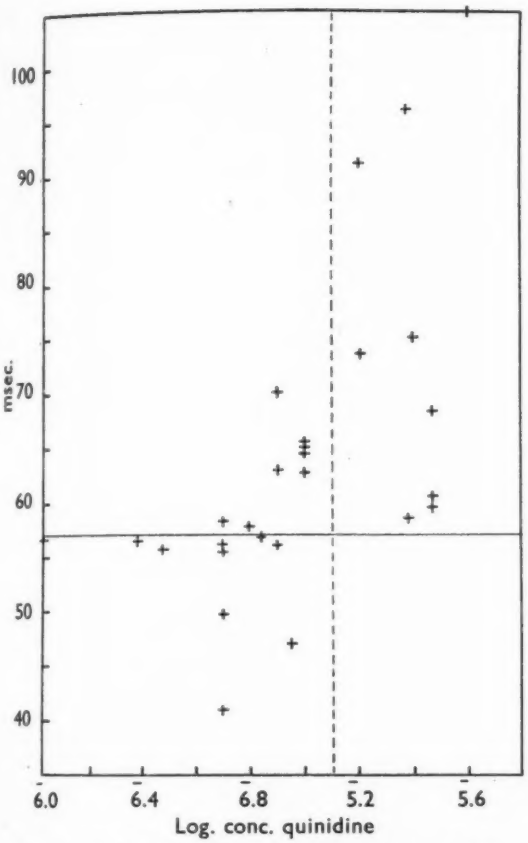

FIG. 6.—Effect of quinidine on the half-time for repolarization. Ordinate: milliseconds. Abscissa: log concentration. At concentrations of below 8 mg./l. the duration was unchanged or reduced. The increase in duration at higher concentrations was partly, or perhaps mainly, due to a reduction in the frequency of stimulation. The horizontal line represents the mean of all the controls. For explanation of vertical broken line, see legend to Fig. 4.

and the height of the overshoot was reduced. There was always also a slowing of conduction velocity.

Refractory Period

The results explain why quinidine, while making no difference to the absolute refractory period, prolongs the effective refractory period. There has long been evidence that the absolute refractory period of cardiac muscle is approximately synchronous with the duration of the action potential (Adrian, 1921 ; Blair, Wedd, and Young, 1941). It has been established (Weidmann, 1955a ; Hoffman *et al.*, 1957) that a second propagated response can take place in a cardiac fibre when repolarization has proceeded to 2/3 of the full resting potential, that is to about −62 mV. in a Purkinje fibre whose resting potential is −96 mV.

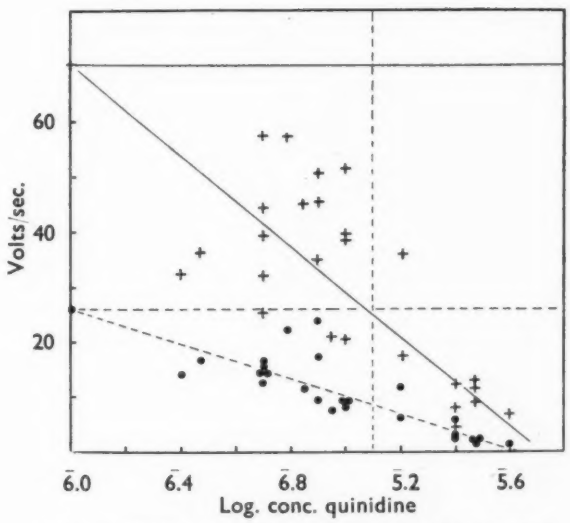

FIG. 7.—Effect of quinidine on the rate of rise of the action potential. Ordinate: volts/sec. Abscissa: log concentration. Crosses and solid line, maximum rate of rise. Circles and dotted line, mean rate of rise calculated from points 2.5 mV. above and 2.5 mV. below the most negative and positive values respectively of the action potential. Horizontal lines, means of all controls.

It is clear, therefore that the terminal phase of repolarization from −62 to −96 mV. could have no influence upon the effective refractory period, since a propagated response can already be elicited when repolarization has reached −62 mV. Hoffman *et al.* (1957) also showed that a local non-propagated response could be produced a little earlier, when repolarization had proceeded to only −45 mV., or half the resting potential, thus accounting for an absolute refractory period which was a little shorter than the effective refractory period.

For propagation to occur a certain minimum rate of change of the membrane potential must occur to overtake accommodation. Very slowly rising currents fail to excite. Weidmann (1955a) showed that the rate of rise of the action potential was a function of the level of the membrane potential at which the action potential "took off." If a stimulus was introduced when the membrane was repolarized to −65 mV., the action potential took off with a rate of rise much faster than if the stimulus arrived when repolarization had proceeded to only −55 mV. Hoffman *et al.* (1957) confirmed the findings of Weidmann (1955a), and showed that the critical rate of rise for propagation occurred when the response took off at about −50 mV. At −45 mV. a local response was evoked, but the rate of rise was insufficient for propagation.

In the presence of quinidine the rate of rise was greatly reduced. The membrane had therefore to repolarize further before the critical take-off point was reached at which the rate of rise was fast enough for propagation. The duration of the terminal phase of repolarization, normally unimportant, thus became significant in determining the effective refractory period. The absolute refractory period was unchanged, because the duration of the half-time for repolarization was not prolonged by quinidine.

Toxic Concentrations

At concentrations of 16 mg./l. and more it was necessary to reduce the frequency of stimulation, which would have increased the conduction velocity and the rate of rise of the action potential in normal atria. But conduction velocity and the rate of rise were further reduced by high quinidine concentrations in spite of the lowered frequency. In several experiments the duration of the action potential was greatly prolonged by quinidine concentrations of 16 mg./l. and more, but this may have been due to the reduced frequency. West (1955) found that the duration of the action potential was prolonged by quinidine: but since his preparations were beating spontaneously, the prolongation could have been due to the slowing of the rate of the pacemaker by quinidine. If, then, it may be assumed that concentrations of 10 mg./l. or less correspond to therapeutic levels of the drug, the mode of action of quinidine cannot be due to a change in the half-time for repolarization. The absence of slowing, and, in some experiments, the acceleration of the first half of the phase of repolarization is of interest, however, in providing an explanation for the observation that the absolute refractory period is unchanged or shortened by quinidine.

Phase of Depolarization

There are at least three ways in which a reduction in the rate of rise of the action potential could be produced. (1) A fall in the concentration differences inside and outside the fibre of the ions which carry the depolarizing current. The overshoot was reduced by moderate concentrations of quinidine, and might be abolished by 25 mg./l. or more. If the depolarizing ion is sodium, this would imply interference with the sodium pump. (2) A fall in the resting potential. This is evidently an unimportant factor in the mode of action of quinidine. It is also improbable that the intracellular concentration of potassium is altered by quinidine, though the total flux might be reduced. (3) An interference with the reaction which precedes or accompanies the flow of depolarizing current. This reaction has a high Q_{10}. The rate of rise is greatly reduced by a lowering of temperature from 36° to 26°, whereas the resting and action potentials are unaffected (Vaughan Williams, 1958). It is certainly a possibility that quinidine interferes directly with this reaction of depolarization.

There is as yet no evidence whether sodium is the ion which carries the current during depolarization in the rabbit auricle. In the cat auricle Burgen and Terroux (1953), and in Purkinje fibres Draper and Weidmann (1951), found that reduction of external sodium reduced the overshoot, but in these experiments sodium chloride was replaced by sucrose and saccharose respectively; so that there was a deficiency in the total ionic strength. In the guinea-pig ventricle, when external sodium was replaced by choline chloride, the overshoot was little affected (Corabœuf and Otsuka, 1956). There is evidence that in some tissues ions other than sodium can enter the cell during depolarization (Fatt and Katz, 1953; Hodgkin and Keynes, 1957; Fatt and Ginsborg, 1958).

At high concentrations of quinidine, the upstroke of the action potential developed a "step." A fast phase of depolarization (Fig. 3) was cut off early, yet depolarization proceeded at a slower rate to an overshoot. It is possible that the depolarization was cut short in the fibre in which the electrode was situated, but continued in distant fibres so that a further depolarization was observed, conducted back to the electrode electrotonically. Against this explanation is the observation that the "stepped" rising phase was observed at all sites penetrated in the presence of high quinidine concentrations, in several different auricles. Another possibility which might be considered is that the early fast phase of the step represents the operation of the sodium-carrier, and that the second slower phase is produced by current carried by other ions. Whatever the explanation for the "step," the early fast phase was accelerated and augmented by ACh, implying a direct action by ACh on the phase of depolarization (Vaughan Williams, 1958). In the experiments of Corabœuf and Otsuka (1956), although the final overshoot voltage reached in low sodium solutions was not reduced, the rate of rise was much slower. The sodium-carrier therefore might normally be the fastest but is not necessarily the only mechanism for transporting depolarizing current.

The problem could be solved by a method with sufficient resolution to measure the flux of ions during the different phases of the action potential,

but measurements of this kind have so far been found possible only in the slowly acting ventricle of the turtle (Wilde, O'Brien, and Bay, 1955). Measurements of average fluxes of ions can give only limited information, because the concentration differences responsible for the resting and action potential heights can remain unchanged over a very wide range of absolute fluxes, as shown by their resistance to temperature change (Burgen and Terroux, 1953; Trautwein, 1953; Trautwein, Gottstein and Federschmidt, 1953; Vaughan Williams, 1958).

In conclusion, it is apparent that the present work has some relevance to the hypotheses of Holland (1957) and Armitage (1957) concerning the rôle of potassium in the mode of action of quinidine in preventing fibrillation. These authors found that lowering the external potassium reduced the depressant effect of quinidine on isolated rabbit atria. It was concluded that quinidine acted by reducing potassium outflux, and so prolonging the duration of the action potential. Holland (1957), however, found that quinidine, even in as high a concentration as 50 mg./l., had by itself only a slight effect on potassium outflux, though its antagonism to ACh could be demonstrated. Burn, Gunning, and Walker (1956), Armitage (1957), and Armitage, Burn, and Gunning (1957) likewise took the view that the effect of changes in external potassium concentration acted mainly by altering the duration of the action potential. It was argued that by raising the external potassium the concentration difference assumed to be responsible for potassium outflux during repolarization was reduced, so that the phase of repolarization was prolonged. Against this must be set the evidence of Weidmann (1955a, 1956) and Carmeliet and Lacquet (1956) that raising the external potassium does not lengthen, but shortens, the cardiac action potential. On the other hand, if it is accepted that a reasonably high level of excitability with an adequate rate of rise of action potential is a prerequisite for the onset of fibrillation, the effects of potassium could be interpreted in another way. Raising the external potassium, by lowering the resting potential, would automatically reduce the rate of rise, and make fibrillation less probable. ACh, by increasing the rate of rise, would make fibrillation more probable. Quinidine reduces the rate of rise, and this action would be opposed by low external potassium, because the latter, by increasing the resting potential, would automatically accelerate the rate of rise.

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EFFECT OF PYRIDINE ALDOXIMES ON RESPONSE OF FROG RECTUS MUSCLE TO ACETYLCHOLINE

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The effects of pyridine-2, 3- and 4-aldoxime methiodide (PAM) on the isotonic contracture of the isolated frog rectus abdominis muscle elicited by acetylcholine have been studied. The potentiation to acetylcholine resulting from incubation with PAM isomers is reversible and may be related to the weak anticholinesterase property of these compounds. Concentrations of the isomers higher than those needed for maximal potentiation produce a reversible block of the muscle to acetylcholine. *p*H studies suggest that cations are responsible for the potentiating and blocking properties of the PAM isomers.

Pyridine-2-aldoxime methiodide (P2AM) is an effective therapeutic compound when used as an adjunct to atropine in the treatment of anticholinesterase poisoning (Askew, 1957; Wills, Kunkel, Brown, and Groblewski, 1957). However, Kewitz and Wilson (1956) reported that lethal doses of P2AM produced death from respiratory paralysis. Holmes and Robins (1955) found that this compound produced neuromuscular block in the isolated phrenic nerve-diaphragm preparation of the rat.

Because of the possible therapeutic importance of P2AM, the effects of this compound and its 3- and 4-isomers on a neuromuscular junction have been further investigated on the isolated frog rectus abdominis muscle.

METHODS

Solutions.—Frog Ringer: NaCl, 0.56; KCl, 0.0075; $CaCl_2$, 0.01; dextrose, 0.2% (w/v). Buffered with 0.02M-tris (hydroxymethyl) amino methane (TRIS) at *p*H 7.2. Oxygen was bubbled through the solution continuously during the experiments. The response of the rectus in Ringer buffered as above has been found comparable with that obtained in the conventional bicarbonate Ringer with the *p*H set by bubbling 95% O_2 and 5% CO_2. Frog Ringer buffered with TRIS possessed the advantage of permitting *p*H studies, with only negligible change in ionic strength.

Eserine salicylate (Merck) was used in a concentration of 2.5×10^{-5}M in frog Ringer. Acetylcholine (ACh) [Merck] was weighed and then dissolved in 5 ml. of cold acetate buffer *p*H 4.5 to yield a concentrated stock solution which was stored at 3° for not longer than one week. Aliquots of the stock were diluted with frog Ringer to the desired concentration immediately before use. The quantities of ACh used are expressed in μg./ml. of the free base.

Pyridine Aldoxime Methiodide Derivatives.—Samples of pyridine 2-, 3-, and 4-aldoxime methiodides were synthesized in the Chemotherapy Branch of Chemical Warfare Laboratories by Dr. Brennie E. Hackley, Jr., and were characterized by melting point and elemental analysis.

Measurement of Isotonic Contraction.—Frogs of either sex weighing 20 to 30 g. were used. The rectus abdominis muscle was dissected out and immersed in a temperature-regulated chamber (25° ± 0.2) of Ringer solution. ACh was added to yield a final volume of 4 ml. in the bath. A 1 min. contracture was recorded. The preparation was then washed four times with frog Ringer for periods of 30 sec. each, and the muscle was allowed to rest 9 min. before the next test solution of ACh was added.

The character of some of these experiments was such that the muscles were not tested for 1 hr. or more—as, for example, during incubation with an anticholinesterase. As the first response after a prolonged inactive period was often different from that produced when the contractures were elicited at the regular time intervals, the response after a longer rest period was always checked by successive measurements performed at 9 min. intervals. Two to three successive measurements generally sufficed to yield an identical rate of contraction (±1 mm./min.) to a constant dose of ACh.

RESULTS

Effect of P2AM upon the Response of the Rectus Abdominis to ACh. Relation to Cholinesterase Activity.—The rate of contracture of the isolated muscle to a given dose of ACh was measured both in the absence of P2AM as a control and then in the presence of the concentrations of P2AM listed in Table I. It may be seen that significant potentiation of the rate of contracture appeared at 1.0×10^{-4}M-P2AM and increased to a maximum at 7.5×10^{-4}M. Further increase in the concentration of P2AM resulted in a decrease in the rate of contracture to well below that of the control at 5.0×10^{-3}M-P2AM. This inhibitory effect on the response could be antagonized by doubling the concentration of ACh; both the potentiating and inhibitory effects were readily reversed on washing.

TABLE I

EFFECT OF P2AM ON THE CONTRACTURE PRODUCED BY ADDITION OF ACETYLCHOLINE TO ISOLATED FROG RECTUS ABDOMINIS MUSCLE

Conc. of P2AM (molar)	ACh Added (μg./ml.)	Rate of Contracture (mm./min.)
0	0·3	16·0
0	0·3	15·5
1.0×10^{-5}	0·3	16·0
1.0×10^{-5}	0·3	16·0
5.0×10^{-5}	0·3	18·0
1.0×10^{-4}	0·3	19·0
2.5×10^{-4}	0·3	24·0
5.0×10^{-4}	0·3	27·5
7.5×10^{-4}	0·3	29·0
1.0×10^{-3}	0·3	28·0
5.0×10^{-3}	0·3	11·5
5.0×10^{-3}	0·6	27·5
0	0·3	16·0
0	0·3	15·5

Measurements of the cholinesterase (ChE) activity (Fleisher, Corrigan, and Howard, unpublished observations) of the contralateral muscle from the same frog in 2.5 and 5.0×10^{-4}M-P2AM show a reduction of 23% and 40% respectively of the ChE activity obtained in the absence of P2AM, thus accounting, at least in part, for the observed potentiation of the rate of contracture at these concentrations of the oxime.

The site of the competitive antagonism between ACh and P2AM was studied further by measuring the responses of the isolated muscle to a constant dose of ACh (0.25 μg./ml.) in the presence of the successively greater concentrations of P2AM noted in Table II. After washing out the P2AM, and again obtaining the control response, the muscle was incubated with 2.5×10^{-5}M eserine for 1 hr. at 25° which completely inactivated the ChE activity. (Fresh muscles incubated with 2.5×10^{-5}M-eserine for periods exceeding 1 hr. showed no ChE activity.) The responses to the same dose of ACh were again obtained with the concentrations of P2AM used before, this time in the presence of 2.5×10^{-5}M-eserine. Table II shows maximum potentiation at 7.5×10^{-4}M-P2AM before incubation with eserine, followed by a decrease in the rate of contracture. After the ChE is wholly inactivated by eserine, the only effect observed in the presence of P2AM is antagonism to ACh, which increases markedly as the concentration of P2AM exceeds 1.0×10^{-3}M.

TABLE II

EFFECTS OF VARYING CONCENTRATIONS OF P2AM IN THE ABSENCE AND PRESENCE OF ESERINE UPON THE RESPONSE OF THE ISOLATED FROG RECTUS ABDOMINIS MUSCLE TO ACETYLCHOLINE

Conc. of P2AM (molar)	ACh Added (μg./ml.)	Rate of Contracture (mm./min.)	
		No Eserine	In 2.5×10^{-5} M Eserine
0	0·25	12	47
1.0×10^{-4}	0·25	14	46
2.5×10^{-4}	0·25	19	43
5.0×10^{-4}	0·25	22	41
7.5×10^{-4}	0·25	23	39
1.0×10^{-3}	0·25	22	34
2.5×10^{-3}	0·25	19	23
5.0×10^{-3}	0·25	13·5	12·5
5.0×10^{-3}	0·50	31	25
0	0·25	13	43
0	0·25	12	45

Effects of p*H upon the Response of the Isolated Rectus Muscle to ACh in the Presence of P2AM or its 3- and 4-isomers.*—The structure of P2AM and its 3- and 4-isomers is such that the ratio of the acid form of the molecule to the salt form would vary considerably with *p*H. It was of interest to study whether this variation in the ratio of anion to cation, produced by changes in the *p*H of the medium, could affect the response of the muscle to ACh. Control responses to ACh were first measured at *p*H 7.2, 8.0, and 8.6 and the rates of contracture varied no more than 2 to 3 mm./min. The effect of *p*H on further contractures produced by the addition of ACh in a final concentration of 0.5 μg./ml. to the incubation medium which contained progressively higher concentrations of P2AM was then studied on the same muscle at *p*H 7.2, 8.0, and 8.6. The results for all three *p*H values are shown in Fig. 1.

Similar studies were performed at *p*H 7.2, 8.0, and 8.6 with P3AM and P4AM respectively. From the known pK_a values for pyridine 2-, 3-, and 4-aldoxime methiodide (Wilson and Ginsberg, 1957) the proportion of the total concentration yielding the maximal rate of contracture which is in the cationic form may be calculated (Table III). Note that the concentration of cation associated with maximal potentiation of response remains roughly the same for P2AM and P3AM

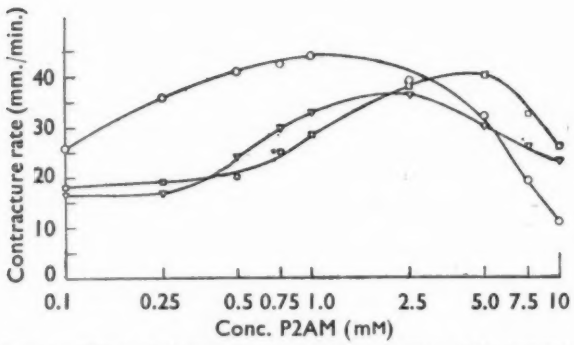

FIG. 1.—Effect of change of *p*H upon the response of the isolated frog rectus abdominis muscle to acetylcholine in the presence of increasing concentrations of P2AM. O *p*H 7.2; ▽ *p*H 8.0; □ *p*H 8.6.

while the total concentration of the compound varies over a fivefold range. The concentration of cation producing maximum response is more variable with P4AM, but still shows considerably smaller variation than the total concentration of the compound. The results suggest that potentiation is strongly influenced by, although not totally dependent on, the cation.

TABLE III

PROPORTION OF CATIONS OF PYRIDINE 2-, 3-, AND 4-ALDOXIME METHIODIDES AT *p*H 7.2, 8.0, AND 8.6

Compound	*p*K	*p*H	% of Cation	Conc. Yielding Maximal Rate of Contracture	
				Total Conc. (mM)	Molar Conc. of Cation (mM)
P2AM ..	8·0	7·2	86	1·0	0·86
		8·0	50	2·5	1·25
		8·6	20	5·0	1·00
P3AM ..	9·2	7·2	99	0·75	0·74
		8·0	94	0·75	0·71
		8·6	80	1·00	0·80
P4AM ..	8·6	7·2	96	0·75	0·72
		8·0	80	0·75	0·60
		8·6	50	2·50	1·25

Fig. 1 also shows a greater depression in the response to ACh for values of P2AM exceeding those required for the maximum contracture when the *p*H is 7.2 than at higher *p*H values. Since the cation is predominant at the lower *p*H of 7.2, an influence of this ion on the blocking of the response to ACh by P2AM is suggested.

DISCUSSION

Wilson and Ginsberg (1955) and Childs, Davies, Green and Rutland (1955) were the first to show the potent ChE-reactivating property of P2AM. The present paper reports other effects produced by this compound at the neuromuscular junction in the frog rectus abdominis muscle, namely an ACh-potentiating effect and an ACh-blocking property. The contracture produced by ACh in the presence of P2AM is therefore the result of these properties. Furthermore, in confirmation of the ineffectiveness of P2AM against eserine *in vivo* (Kewitz, Wilson and Nachmansohn, 1956), control experiments demonstrated no reactivating effects of P2AM against eserine-inactivated ChE of the isolated rectus muscle. Consequently, if the potentiating effect of P2AM is minimized by superimposition of the greater one produced by eserine, the blocking action of P2AM becomes predominant and can be shown to occur at concentrations where Holmes and Robins (1955) observed neuromuscular blocking action by this compound.

Both the potentiating and ACh-blocking potency of P2AM decrease with increase of *p*H from 7.2 to 8.6 concurrent with an increase in the proportion of anionic component from 14 to 80%. However, it is significant that at *p*H 7.2 there is a significant proportion of the anionic component of P2AM along with the cationic component which is presumed to contribute to reactivation by being bound or attracted to the anionic site on the ChE enzyme of the muscle. Among the three isomeric compounds, the proportion of available anionic component at physiological *p*H values varies as P2AM>P4AM>P3AM. The relative reactivating ability falls off even more rapidly at *p*H 7.2 (Fleisher, Howard and Corrigan, unpublished observation) indicating that either a certain minimal proportion of anionic component must be available for effective reactivation, or that other factors are also involved in the reaction of these compounds with inhibited ChE of the rectus muscle.

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POTENTIATION OF THE RESPONSE OF FROG RECTUS MUSCLE TO ACETYLCHOLINE BY *ISO*PROPYL METHYL PHOSPHONOFLUORIDATE AND ITS MODIFICATION BY PYRIDINE-2-ALDOXIME METHIODIDE

BY

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Pyridine-2-aldoxime methiodide (P2AM) was used to study the relation between the recovery of cholinesterase activity of isolated frog rectus abdominis muscle and the change of isotonic response to acetylcholine after previous treatment with the anticholinesterase, *iso*propyl methyl phosphonofluoridate (sarin). Addition of P2AM to muscle which had been incubated with sarin produced an 88% decrease in potentiation to acetylcholine. This was accompanied by 71% and 35% recoveries of the cholinesterase activity of the intact and finely ground muscle respectively compared with controls from the contralateral muscle. Following pre-treatment with sarin, a two-hour rinsing with acetylcholine (3 μg./ml.) produced a 61% decrease in potentiation to acetylcholine accompanied by 24% and 4.5% recoveries of cholinesterase activity in intact and in ground muscle respectively. Since control experiments showed absence of uncombined sarin in the muscle after rinsing with acetylcholine solution, the results indicate a greater effectiveness of P2AM and acetylcholine in reactivating superficially situated cholinesterase of the frog rectus abdominis as compared with enzyme within the interior of the muscle.

Cohen and Posthumus (1955) observed a partial decrease in the response of the isolated frog rectus abdominis muscle to acetylcholine (ACh) upon prolonged washing following previous incubation with anticholinesterases. However, no corresponding reversal of the inactivation of the cholinesterase (ChE) activity could be demonstrated in finely ground preparations of these muscles referred to subsequently as "homogenates." In our laboratory, prolonged washing (up to 24 hr.) of sarin-incubated muscles with buffered frog Ringer did not yield any significant lowering of the potentiated response to ACh which could be ascribed to a true reversal. The partial lowering observed in some of our preparations appeared due to fatigue, or to some non-specific action of the anticholinesterase limiting the contraction of the muscle. This conclusion was based on the observation that muscles showing such a reduced response after washing with Ringer solution could not have their potentiated response to ACh restored by a second incubation with anticholinesterase. It was decided, therefore, to attempt a much greater degree of reversal of potentiation by treatment of the maximally potentiated muscle with pyridine-2-aldoxime methiodide (P2AM) shown by Wilson and Ginsberg (1955) and Childs, Davies, Green and Rutland (1955) to be a potent reactivator of inhibited ChE.

METHODS

*Iso*propyl methyl phosphonofluoridate (sarin) was obtained from the Field Toxicology Branch of Medical Research Directorate. The purity was 95% or better according to an analysis of acidity.

For other materials, and for the method of measuring isotonic contraction, see Fleisher, Howard, and Corrigan (1958).

Measurement of Cholinesterase Activity. — ChE activity was determined by a bioassay procedure using the isotonic responses of an eserine-treated rectus abdominis muscle from another frog as a measure of the ACh remaining in the mixture of substrate and the experimental muscle after various periods of incubation. The experimental muscle was blotted gently upon removal, weighed to the nearest mg., and placed in 1.9 ml. of frog Ringer at *p*H 7.2. At zero time, 0.1 ml. of substrate was added and the mixture shaken at 120 oscillations/min. in a water bath at

25°. Serial aliquots of 0.1 ml. each were removed at known time intervals and added to the bath containing the muscle. The contractures so elicited were compared alternately with those given by the addition of known standard amounts of ACh. The logarithms of the concentrations of ACh thus estimated were plotted against time. Since the concentration of ACh used in the incubation mixture was always 1.25×10^{-4} M or lower, first order kinetics could be applied to the calculation of enzyme activity (Augustinsson, 1950). From the linear plots thus obtained the time (in min.) for hydrolysis of one half the initial amount of substrate ($t_{1/2}$) was calculated. The first order rate constant was obtained by substitution in the formula: $k = \frac{0.693 \times 60_{hr.} - 1}{t_{1/2}}$ (Neilands and Stumpf, 1955) and ChE activity was expressed as k/mg. of tissue.

The ChE activity of finely ground muscle was determined in a final tissue concentration of 2.5 mg./ml. (unless otherwise noted) after grinding the entire muscle for 2 min. in a Potter-Elvehjem homogenizer powered by a cone-driven stirring motor. The substrate concentration was the same as that used for determining the ChE activity of intact muscle. Control studies, using the same amount of homogenate without added substrate, showed the absence of ACh when the homogenate was added to the eserine-treated assay muscle. Concurrent controls run with substrate in the absence of tissue showed no non-enzymatic hydrolysis under the conditions used (25° and *p*H 7.2).

RESULTS

Control Studies on the ChE Activity of Intact and Finely Ground Frog Rectus Abdominis Muscle

The need to refer changes in ChE activity of one rectus muscle following experimental treatment to the contralateral muscle required a preliminary control experiment. For this, the ChE activity of each intact muscle was determined followed by washing, grinding and measurement of the ChE activity of the homogenate. Results are given in Table I. It may be noted that, with the exception of the homogenates from frog No. 2, the ChE activity of one muscle of a pair, whether intact or as a homogenate, was within 10% of the other. In addition, the rate of hydrolysis by the intact muscle averaged less than 1/20 of that of an equal weight of finely ground tissue, pointing to the necessity of the sensitive bioassay technique used in the determination of ChE activity.

TABLE I

CHOLINESTERASE ACTIVITY OF INTACT AND GROUND-UP CONTRALATERAL FROG RECTUS ABDOMINIS MUSCLES

(Activity expressed as k/mg. of tissue fresh weight)

Frog No.	Intact Muscle			Ground Muscle		
	Left	Right	Difference %	Left	Right	Difference %
1	0·014	0·015	7	0·520	0·490	6
2	0·013	0·012	8	0·260	0·297	12
3	0·009	0·010	10	0·287	0·260	9
4	0·010	0·010	0	0·333	0·362	8
5	0·011	0·010	9	0·255	0·238	7
6	0·009	0·009	0	0·347	0·347	0
7	0·013	0·014	7	0·347	0·314	9
8	0·020	0·020	0	0·347	0·370	6
	Mean % Difference 5±1·4 (S.E.)			Mean % Difference 7±1·2 (S.E.)		

The Relation Between the Reduced Response of Sarin-treated Muscle and the Restoration of ChE Activity with P2AM

On the basis of the previous control study, the following experimental procedure was adopted. One muscle was used as a control for ChE activity, first as an intact muscle, then as a homogenate. Concurrently, the concentration of ACh required to elicit a given response in the experimental muscle was determined before and after incubation with 5.0×10^{-7} M sarin for 1 hr. The ratio of the two concentrations of ACh required was used to obtain the approximate potentiation ratio. The muscle was then incubated for 8 min. with 2.0×10^{-4} M P2AM and the concentration of ACh required to elicit the original response was again determined enabling the calculation of the change in the potentiation ratio. After washing with Ringer, the ChE activity of the intact experimental muscle, and of a homogenate prepared from that muscle, was determined under conditions identical with that of the control, with the exception that 5 mg. of tissue/ml. of incubation mixture was used in the determination of the ChE activity of the experimental homogenate to ensure sufficient activity for accurate measurement.

The first order rate constants obtained from the linear plots shown in Fig. 1 for both control and experimental preparations are given in Table II.

From Table II, the % decrease in the potentiation ratio following the administration of P2AM is calculated as:

$$\frac{\text{Potentiation ratio after sarin minus potentiation ratio after P2AM}}{\text{Potentiation ratio after sarin}} \times 100 = 88.$$

It may be noted that the observed recovery of 71% in the ChE activity of intact muscle is more closely related than that of the homogenate (35%) to the 88% decrease in the potentiation ratio following treatment with P2AM.

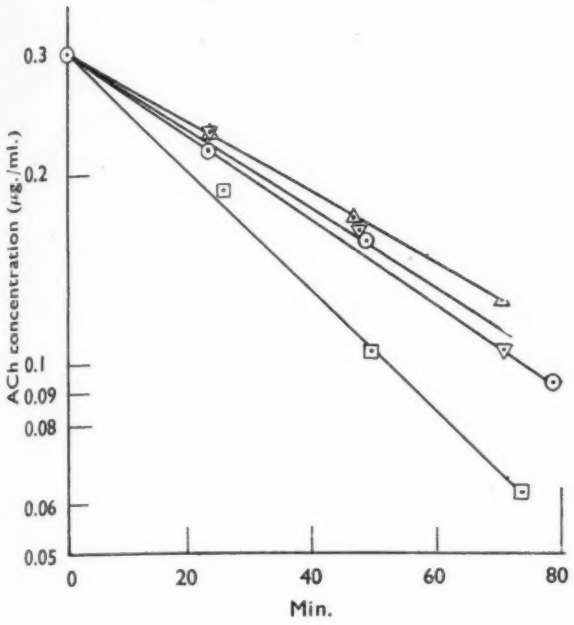

FIG. 1.—Cholinesterase activity of intact and homogenized frog rectus abdominis muscle (previously incubated with sarin) after treatment with P2AM. The lines from below upwards represent results with control homogenate (2·5 mg./ml.); initial activity of intact muscle; homogenate (5 mg./ml.) after P2AM; intact muscle after P2AM.

TABLE II

EFFECT OF P2AM ON CHOLINESTERASE ACTIVITY AND ON THE RESPONSE OF ISOLATED FROG RECTUS ABDOMINIS MUSCLE TO ACETYLCHOLINE FOLLOWING INCUBATION WITH SARIN

The approximate potentiation ratio=ACh required for initial response (μg./ml.)/ACh required following experimental treatment (μg./ml.); (a) negligible refers to a series of 4 inhibition controls obtained by measuring the ChE activity of sarin-incubated muscles (both as intact muscles and as ground preparations) which showed no significant ChE activity during the first 30 min.

Condition Studied	Muscular Response			Cholinesterase Activity	
	Rate of Contracture (mm./min.)	ACh Required (μg./ml.)	Approx. Potentiation Ratio	Intact Muscle k/mg.	Homogenate k/mg.
Before sarin	30·0	3·0	1·0	0·016	0·259
After sarin	30·0	0·2	15·0	Negligible (a)	
After P2AM	29·5	1·8	1·7	0·012	0·090

Effects of ACh upon the ChE Activity of Frog Rectus Muscle after Incubation with Sarin

To determine whether ACh by itself could produce any significant restoration of ChE in the muscle after incubation with sarin, the initial ChE activity of three muscles together was determined in 4 ml. of incubation mixture. The muscles were then washed with four portions of Ringer, and suspended in 5.0×10^{-7} M sarin for 1 hr. (sufficient to produce complete inactivation of ChE). After 12 washes over a 30 min. period with Ringer, the muscles were then reincubated with ACh as before and the ChE activity was determined.

The three muscles were again washed with four portions of Ringer, homogenized together, and the ChE activity of the homogenate determined in a final tissue concentration of 25 mg./ml. of incubation mixture, but with the same substrate concentration as before. The ChE activity of each preparation and that of a control homogenate made from the three corresponding contralateral muscles from the three frogs used to supply the experimental preparation is shown in Fig. 2. It

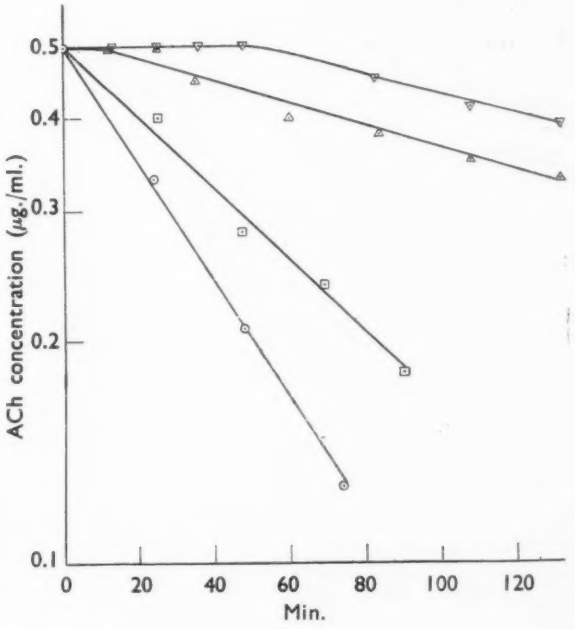

FIG. 2.—Cholinesterase activity of intact and homogenized frog rectus abdominis muscles after incubation with sarin. The lines from below upwards represent results with 3 intact muscles before sarin; control homogenate (2·5 mg./ml.); 3 intact muscles after sarin; experimental homogenate (25·0 mg./ml.). For further explanation, see text.

may be noted that during the first 30 min. of incubation with ACh following incubation with sarin, no significant ChE activity of the intact muscles could be demonstrated; however, after this time, the hydrolysis of ACh begins to take place at a rate equal to 24% of the initial activity. The measurements of recovery are based on the first order rate constants 0.007 $hr.^{-1}$ $mg.^{-1}$ and 0.002 $hr.^{-1}$ $mg.^{-1}$ found for the initial and experimental intact muscle activities respec-

tively; and 0.264 hr.$^{-1}$ mg.$^{-1}$ and 0.012 hr.$^{-1}$ mg.$^{-1}$ for the control and experimental activities of the ground muscle respectively. All values for $t_{1/2}$ were computed from the linear portions of the curves in Fig. 2. The homogenate from the experimental muscle required 80 min. of incubation with ACh before measurable ChE activity developed. Thereafter, the hydrolysis rate was equal to 4.5% of the corresponding control.

Evidence for Absence of Sarin in Frog Rectus Muscle Washed after Incubation with Sarin

This study was performed to determine whether the very low activity of the homogenate prepared from the experimental muscles in the previous study resulted from the presence of uncombined anticholinesterase.

Three experimental muscles were treated with sarin, followed by washing in a manner identical with the previous study. Then each muscle was divided into halves longitudinally. One half muscle from muscle 1 was grouped with one half from muscles 2 and 3 so that two corresponding sets of half muscles were obtained. The ChE activity of one set of halves was then determined after grinding up in a final tissue concentration of 25 mg./ml. of incubation mixture. The second group of three halves was homogenized in a control homogenate previously prepared from the three corresponding contralateral muscles, a procedure similar in principle to that used by Koelle and Steiner (1956). However, 10 times as much experimental tissue was taken as was present in the control homogenate, so that, if free inhibitor were present, a sufficient excess of it would be available during homogenization for complete inactivation of the ChE activity of the control. Results are given in Table III. Note that the ChE activity of the control, homogenized with 10 times as much washed sarin-treated muscle, is within 95% of its original activity indicating absence of significant amounts of uncombined anticholinesterase.

TABLE III

EVIDENCE FOR ABSENCE OF SARIN IN FROG RECTUS ABDOMINIS MUSCLES WASHED AFTER TREATMENT WITH SARIN

Condition Tested	Tissue Conc. (mg./ml.)	Cholinesterase Activity	
		Half Time (min.)	Rate Constant k/mg.
(1) Not treated with sarin	2·5	70	0·594
(2) Treated with sarin ..	25·0	98	0·424
(3) Combined homogenates	25·0+2 5	42	0·990
(3)–(2)			0·566

The Relation Between the Diminished Response to ACh of Sarin-treated Muscles and the Restoration of ChE Activity with ACh

It has been demonstrated that a partial return of ChE activity of intact muscle could be accomplished if sarin-treated muscles were incubated with ACh alone. When muscular responses were measured following incubation with ACh in a concentration of 20 μg./ml., a lowering of neuromuscular response was obtained, but the results were open to question because of possible injury to receptor sites in the muscle by this concentration of ACh.

It was, therefore, decided to wash the sarin-treated muscle with an ACh solution containing 3 μg. ACh/ml., a level which in control studies produced no injurious effect upon the neuromuscular response. The effects of a 2 hr. rinse with 1,000 ml. of an ACh solution containing 3 μg./ml. of ACh upon the responsiveness of sarin-treated muscle is shown in Table IV.

TABLE IV

EFFECT OF WASHING WITH ACh ON THE RESPONSE OF FROG RECTUS ABDOMINIS MUSCLE TO ACh AND ON CHOLINESTERASE ACTIVITY FOLLOWING PREVIOUS INCUBATION WITH SARIN

During the 2-hour wash with ACh no tension was placed upon the muscle. For cholinesterase determinations on sarin-treated muscle see Table II. In this study, determinations could be performed on the experimental muscle only after the wash with ACh.

Condition Studied	Muscular Response			Cholinesterase Activity	
	Rate of Contracture (mm./min.)	ACh Required (μg./ml.)	Potentiation Ratio	Intact Muscle k/mg.	Homogenates k/mg.
Before sarin ..	19·5	1·00	1·0	0·017	0·554
After sarin ..	19·5	0·08	12·5		
After washing with ACh ..	19·5	0·20	5·0	0·004	0·031

Studies of the ChE activity of this muscle following the ACh wash were done both on the intact muscle and on a homogenate prepared from it. The results were compared with the ChE activity of the contralateral muscle which had not been exposed to sarin (Table IV). It may be seen that a reduction in the potentiation ratio of 61% is accompanied by a recovery of 24% of the ChE activity of the intact muscle. The homogenate prepared from the experimental muscle showed only 5.6% of the ChE activity of the corresponding control.

DISCUSSION

The disparity between the ChE activity of intact muscle and that of homogenates following treatment with P2AM may result from the relatively

greater effectiveness of the quaternary amine in reactivating the more superficially situated ChE enzyme than that within the interior of the muscle. In consequence homogenization leading to a determination of the ChE of the whole tissue yields a value for the ChE activity which is affected by the lesser degree of reactivation of the internally situated enzyme which accounts for over 95% of the ChE activity (Tables I and II).

However, the closer association between recovery after sarin-induced poisoning of the ChE of the neuromuscular junction, where most of the surface ChE of the muscle is located (Couteaux and Nachmansohn, 1940), and the degree of return of the normal responsiveness to ACh after treatment with P2AM (Table II), suggests that it is the activity of the surface enzyme which is important. This view is supported by the work of Koelle (1957) with autonomic ganglia. After poisoning with an anticholinesterase most of the ChE of the ganglia is inactivated; treatment by intravenous injection of P2AM alters very little the total ChE of ganglia, but does induce a nearly complete regeneration of ChE activity at the periphery of the ganglion cell as evidenced by histochemical demonstration of ChE.

24% of the initial ChE activity of surface enzyme (intact muscle) returns if sarin-treated muscles are incubated with ACh alone, as compared with 4.5% of the corresponding activity in homogenates. This suggests that ACh itself may bring about a partial displacement of the anticholinesterases from the more important surface sites of muscle, but only negligible displacement from the bulk of internally situated ChE. The possibility that the very low activity of ChE in homogenates is due to a secondary inactivation by uncombined ChE inhibitor would appear to be excluded by the results recorded in Table III.

The latent period of 30 min. and 80 min. required for the sarin-treated intact muscle and homogenate respectively to develop measurable enzymatic activity in the presence of ACh (Fig. 2) may be explained in part by the lesser effectiveness of ACh compared to P2AM in reactivating the surface ChE of intact muscle. It is of interest that the 2-hour wash with an ACh solution containing 3 μg./ml. of ACh yielded a preparation whose activity followed first order kinetics immediately after the addition of substrate: that is, no latent period was required to develop measurable ChE activity. Of further interest was the finding that incubation with ACh in concentrations of 20 μg./ml. for 156 min. (Fig. 2) yielded approximately the same recovery of ChE as a 2-hour incubation with ACh in concentrations of 3 μg./ml. This observation suggests that only part of the available ChE activity of muscle may be reactivated with ACh under the experimental conditions. Yet the recovery of 24% of the surface ChE led to a lowering of 61% in the potentiated response of the muscle to ACh (Table IV). Since this relatively small recovery of ChE activity promoted a large degree of return toward normal function, one may interpret these observations as suggesting a possible mechanism for the spontaneous recovery of neuromuscular function observed by McNamara, Murtha, Bergner, Robinson, Bender, and Wills (1954) following administration of anticholinesterases.

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THE INHIBITORY ACTION OF MORPHINE ON THE CONTRACTION OF THE LONGITUDINAL MUSCLE COAT OF THE ISOLATED GUINEA-PIG ILEUM

BY

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Morphine (0.05 to 0.1 μg./ml.) markedly inhibited the contractions of the isolated guinea-pig ileum caused by nicotine, barium, and 5-hydroxytryptamine while the actions of acetylcholine, carbachol, and histamine were affected only a little. Atropine (0.025 to 0.05 μg./ml.) had a similar effect, in addition to its known effects on acetylcholine and carbachol contractions. Morphine had no additional effect on the inhibitory action of atropine, while in the presence of morphine, atropine had a significant additional inhibitory action only on the contraction caused by nicotine.

The action of barium was complex. It caused contractions of a rhythmic type alternating with relaxations, a pattern which is similar to that produced by the emptying phase of the peristaltic reflex; these oscillations were inhibited by hexamethonium. On the other hand, that part of the sustained barium contraction which was inhibited by morphine may be explained by an action on the nerve cells innervating the muscle fibres (motor neurones). Similarly, the morphine-sensitive component of the 5-hydroxytryptamine (5-HT) contraction was probably also due to an action on the motor neurones. The morphine-insensitive contractions of barium, 5-HT, and nicotine were believed to be caused by a direct action on the muscle fibres.

The morphine inhibitors, nalorphine and levallorphan, had different effects with the different agonists. Their morphine-like action was particularly pronounced on the effect of 5-HT, which they antagonized, while their morphine-protecting action was most strongly present on the effect of nicotine.

In the isolated guinea-pig ileum, small concentrations (0.05 to 0.1 μg./ml.) of morphine inhibit both the preparatory and the emptying phases of the peristaltic reflex (Trendelenburg, 1917; Schaumann, 1955; Kosterlitz and Robinson, 1955, 1957). From the evidence available so far, it would appear that the inhibition takes place on the motor side of the reflex arc since drugs which cause contraction of the longitudinal muscle coat by stimulating the nervous structures of the efferent path are also antagonized by morphine. This was established for nicotine by Schaumann (1955), for 5-hydroxytryptamine by Kosterlitz and Robinson (1955) and Gaddum and Picarelli (1957) and for barium ions by Kosterlitz, Robinson, and Taylor (1957). In this paper the mechanism of morphine inhibition is subjected to further analysis.

METHODS

The contraction of the longitudinal muscle layer was recorded either isotonically by a conventional light lever or isometrically by a condenser myograph or a mechano-electrical transducer (Innes, Kosterlitz, and Robinson, 1957).

The bath fluid was Tyrode solution with a low $MgCl_2$ content (0.01 g./l.), aerated with O_2. There were intervals of 3 min. between the individual experimental procedures such as eliciting the reflex or the addition of drugs to the bath fluid (40 ml.), which was renewed at least every 6 min. to prevent a significant rise of the initial *p*H of 7.8.

The distal end of the guinea-pig ileum (7 to 8 cm.) was used after discarding a length of 10 cm. nearest to the ileo-caecal valve. The ileum was set up with an initial tension of 1 g. 1 to 2 hr. before the beginning of the experiment. Occasionally a preparation stored at 4° for 24 hr. was used.

The doses of the drugs are given in weight of base for acetylcholine chloride, carbachol (carbaminoylcholine chloride), histamine phosphate, 5-hydroxytryptamine creatinine sulphate (5-HT); in weight of salt for hexamethonium iodide, nicotine bitartrate, barium chloride, morphine chloride, atropine sulphate and (−)-hyoscyamine sulphate.

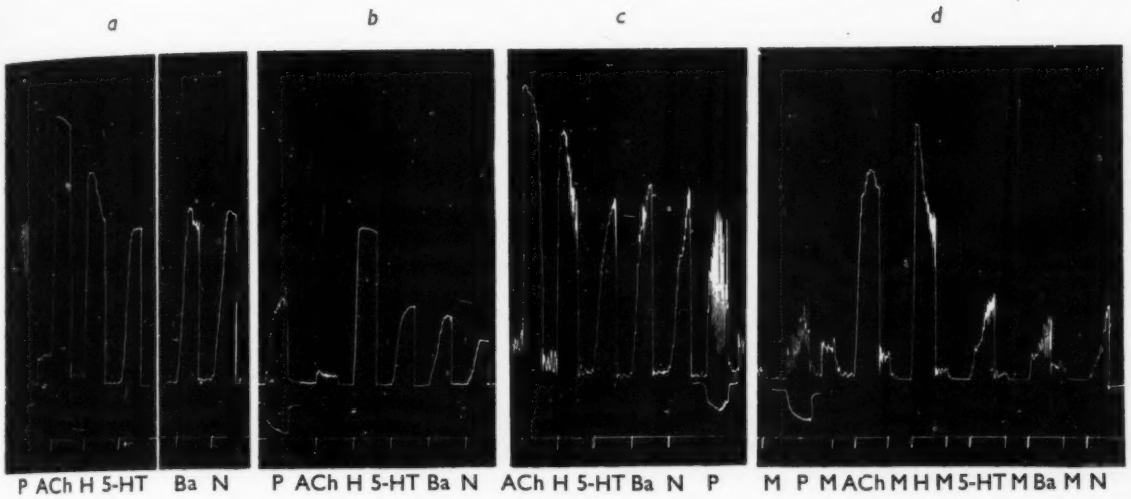

FIG. 1.—The effects of (−)-hyoscyamine and of morphine on isotonic contractions of the longitudinal muscle coat of the isolated guinea-pig ileum. The ileum had been stored at 4° for 24 hr. ***a***, controls. ***b***, in the presence of (−)-hyoscyamine in a concentration of 0.0005 μg./ml. for 53 min. and of 0.001 μg./ml. for 17 min. before ***b***. ***c***, 3½ hr. after washing out (−)-hyoscyamine. ***d***, 18 min. after ***c***, each test 30 sec. after addition of M, morphine (0.05 μg./ml.). P, intra-intestinal pressure raised by 2 cm. H_2O. ACh, acetylcholine (0.0013 μg./ml.). H, histamine (0.0038 μg./ml.). 5-HT, 5-hydroxytryptamine (0.015 μg./ml.). Ba, barium (38 μg./ml.). N, nicotine (1.3 μg./ml.).

RESULTS

Single-dose Experiments Showing the Inhibitory Action of Morphine and (−)-Hyoscyamine

When morphine in a concentration of 0.05 to 0.1 μg./ml. had been present in the bath for 30 sec., the contractions caused by acetylcholine or histamine were depressed only a little or not at all. On the other hand, the contractions due to distension, 5-HT, barium or nicotine were inhibited (Figs. 1*d* and 2). (−)-Hyoscyamine (0.001 μg./ml.) present in the bath fluid throughout the testing period caused a similar depression of the contractions after distension, 5-HT, nicotine and barium but not histamine; the acetylcholine contraction was, of course, inhibited (Fig. 1*b*). While the recovery after hyoscyamine was slow, the inhibitory action of morphine was more transient, recovery usually taking place in less than 15 min.

The contraction caused by barium was somewhat reduced by hexamethonium (25 μg./ml.), a fact already described by Toh (1951), but morphine was a much more powerful inhibitor (Fig. 2). It would appear that the main effect of hexamethonium on the barium contraction was the suppression of large alternating contractions and relaxations of the longitudinal muscle. Visual observation showed these to be associated with contraction of the circular muscle coat of the type found in the peristaltic reflex after intestinal distension.

The Effects of Morphine and Atropine on the Dose/Response Curves of Acetylcholine, Carbachol, Histamine, 5-HT, Barium, and Nicotine

Dose/response curves were obtained for the tensions developed in isometric contractions of the longitudinal muscle layer. In these experiments,

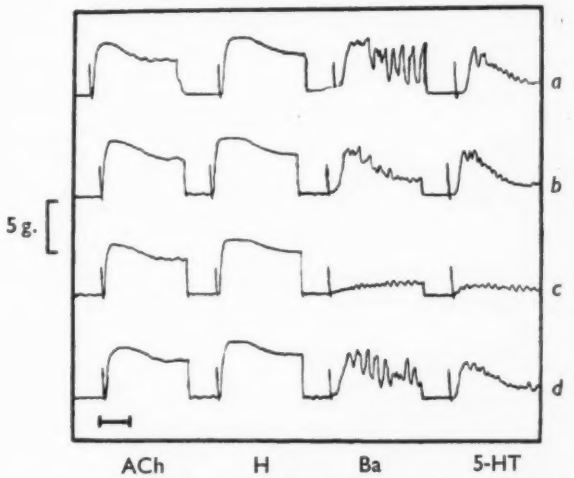

FIG. 2.—The effects of hexamethonium and morphine on isometric contractions of the longitudinal muscle. ***a***, controls. ***b***, 27 min. after addition of hexamethonium (25 μg./ml.). ***c***, 81 min. after addition of hexamethonium and 27 min. after addition of morphine (0.025 μg./ml.). ***d***, 27 min. after washing out. ACh, acetylcholine (0.01 μg./ml.). H, histamine (0.025 μg./ml.). Ba, barium (50 μg./ml.). 5-HT, 5-hydroxytryptamine (0.025 μg./ml.). Time, 10 sec.

the antagonists morphine and atropine were left in the bath fluid throughout the testing periods.

Usually as small a dose as 0.05 μg./ml. of morphine produced the maximum inhibition of nicotine, barium, and 5-HT contractions, doses up to 20 μg./ml. being no more effective (Fig. 3). In most experiments the actions of acetylcholine, carbachol, and histamine showed only little permanent depression; when a more pronounced inhibition was observed, this was smaller than the inhibition of 5-HT, barium, and nicotine (Fig. 4 and Table I). Only occasionally, there was a severe but transient depression of acetylcholine or carbachol. For instance, in the experiment shown in Fig. 3, the carbachol contractions were depressed during the first 27 min. after addition of morphine in a concentration of 0.05 μg./ml. and recovered during the next 27 min. with morphine still present in the same concentration.

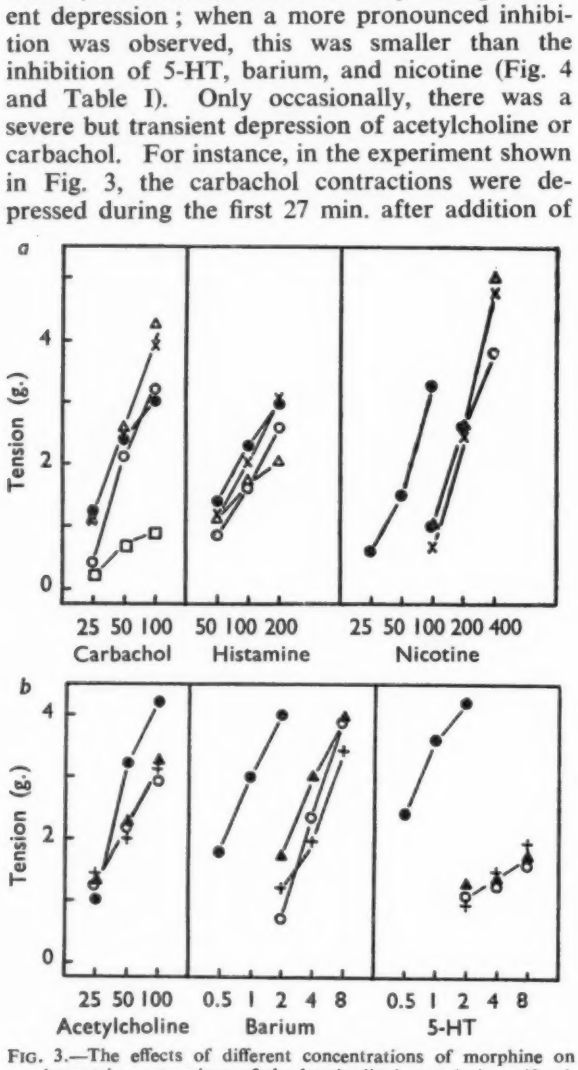

FIG. 3.—The effects of different concentrations of morphine on isometric contractions of the longitudinal muscle in a 40 ml. bath. Two pieces of ileum from two animals were used, *a* without and *b* with hexamethonium (25 μg./ml.). *a*, ● controls. After morphine: □ 3 to 27 min. after 0.05 μg./ml. (carbachol only); O 3 to 54 min. (in the case of carbachol 30 to 54 min.) after 0.05 μg./ml. ✕ 3 to 54 min. after 0.15 μg./ml. △ 3 to 54 min. after 0.45 μg./ml. *b*, ●, controls. After morphine: O, 3 to 54 min. after 0.05 μg./ml. + 3 to 54 min. after 1 μg./ml. ▲, 3 to 54 min. after 20 μg./ml. Each point represents usually but not always the mean of two observations. In the cases of carbachol, histamine and acetylcholine the abscissae are in ng. With barium the abscissa is in mg. while with 5-HT and nicotine in μg.

TABLE I

RATIOS OF DOSES OF ACETYLCHOLINE, CARBACHOL, HISTAMINE, BARIUM, NICOTINE, AND 5-HYDROXYTRYPTAMINE CAUSING EQUAL CONTRACTIONS OF GUINEA-PIG ILEUM IN THE PRESENCE AND ABSENCE OF MORPHINE

The concentration of morphine used was 0·05 to 0·1 μg./ml. In 3 other experiments with 5-HT the dose ratios were >16, >150, >150.

Agonist	No. of Obs.	Dose Ratio	
		Mean	Range
Acetylcholine	16	1·1	0·7–1·4
Carbachol	11	1·4	1·0–3·1
Histamine	9	2·2	1·3–4·4
Barium	15	5·0	2·3–9·0
Nicotine	10	7·0	4·0–15
5-HT	12	23	9–65

A tolerance to morphine, which has been demonstrated by Schaumann (1955) and Paton (1957), did not develop under the conditions of these experiments although each concentration of morphine was maintained for 54 min. After the first application, the dose of morphine was raised

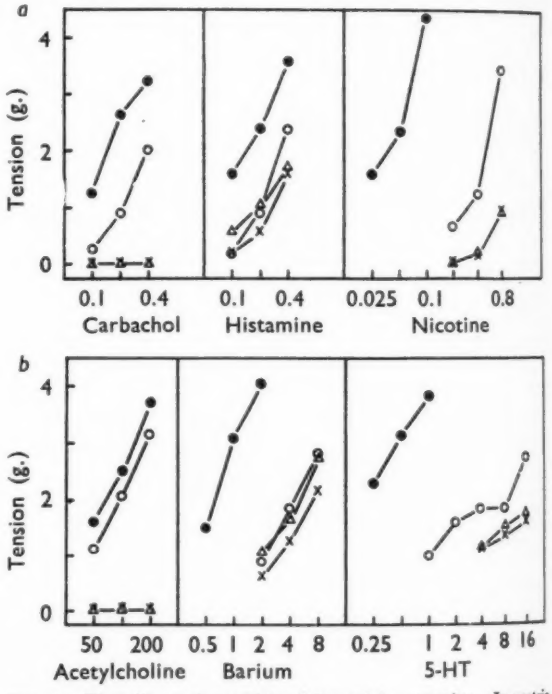

FIG. 4.—The effects of morphine followed by atropine. Isometric contractions of the longitudinal muscle in a 40 ml. bath. Two pieces of ileum from the same animals were used, *a* without and *b* with hexamethonium (25 μg./ml.). ● controls. O 3 to 54 min. after morphine (0.1 μg./ml.). ✕ 3 to 54 min. after morphine (0.1 μg./ml.) and atropine (0.0125 μg./ml.). △ 3 to 54 min. after morphine (0.1 μg./ml.) and atropine (0.025 μg./ml.). Each point is the mean of two observations. Units of abscissae for acetylcholine ng., for carbachol, histamine and 5-HT μg. and for nicotine and barium mg.

twice so that the gut was in contact with morphine for a total of 162 min. (Fig. 3). However, occasionally observations were made which were possibly best explained by partial tolerance. Thus, in one experiment, after repeated applications, morphine lost its inhibitory effect on the nicotine contractions, while the contractions due to intestinal distension or to barium were still morphine-sensitive.

After morphine had been present in the bath for 54 min., addition of atropine (0.013 to 0.025 μg./ml.) reduced the residual contractions of nicotine and 5-HT somewhat but had scarcely any effect on barium (Fig. 4). The additional effect of atropine on the nicotine contraction was always more marked than on that of 5-HT. Conversely, when atropine (0.05 μg./ml.) was applied first, morphine (0.1 μg./ml.) had no additional inhibitory action (Fig. 5).

As was shown above (Fig. 2), hexamethonium depressed the action of barium in the absence of morphine; however, the residual morphine-insensitive barium contraction was unaffected by hexamethonium (Fig. 6). On the other hand, the nicotine contraction was further depressed by hexamethonium after it had been inhibited by a maximal dose of morphine.

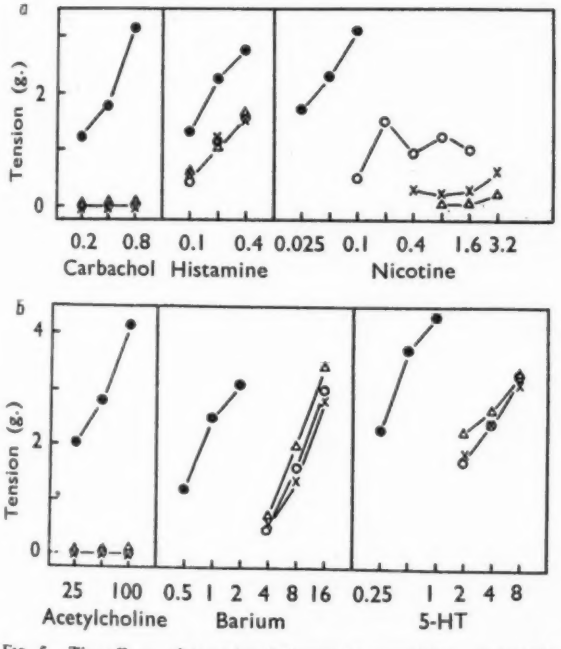

FIG. 5.—The effects of atropine followed by morphine. Isometric contractions of the longitudinal muscle in a 40 ml. bath. Two pieces of ileum from two animals were used (*a* and *b*). ● controls. O 3 to 54 min. after atropine (0.0125 μg./ml.). X 3 to 54 min. after atropine (0.05 μg./ml.). △ 3 to 54 min. after atropine (0.05 μg./ml.) and morphine (0.1 μg./ml.). Each point is the mean of two observations, except the points for nicotine after the first dose of atropine. Units in abscissae for carbachol, histamine, and 5-HT are μg., for acetylcholine ng. and nicotine and barium mg.

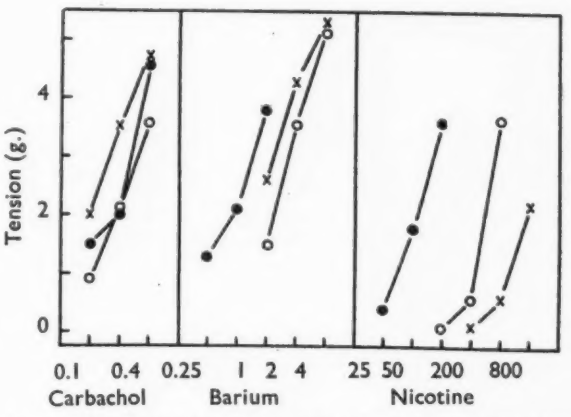

FIG. 6.—The effects of hexamethonium in the presence of morphine. Isometric contractions of the longitudinal muscle in a 40 ml. bath. ● controls. O 3 to 54 min. after morphine (0.1 μg./ml.). X 3 to 54 min. after morphine (0.1 μg./ml.) and hexamethonium (25 μg./ml.). Each point is the mean of two observations. Units of abscissa, for carbachol and nicotine μg. and for barium mg.

Although the dose/response curves were incomplete in that the maximum effect was never reached, an approximate calculation of the ratios of doses of equal potency in the presence and absence of morphine was made, in order to obtain a comparison with the results reported by Gaddum and Picarelli (1957). Whenever possible the dose ratios were determined from the values at the centre of the dose/response curves; however, when the slope of the curve was less steep, due to the addition of the antagonist, it was sometimes found necessary to compare the smallest dose before with the largest dose after the addition of the antagonist. For this reason, the estimates given can be used as a rough guide only. It was found that, in order to obtain the same effects in the presence of morphine (0.05 to 0.1 μg./ml.) as in its absence, the original concentration of acetylcholine, carbachol, and histamine had to be increased by a factor of between 1 and 2, while this factor varied between 5 and 23 for barium, nicotine, and 5-HT (Table I). A similar calculation for atropine (0.025 to 0.05 μg./ml.) as antagonist gave the following factors: histamine 2.8, barium 6, nicotine 160, and 5-HT 19.

The Effects of the Morphine Inhibitors, Nalorphine and Levallorphan

In the intact animal, the morphine inhibitors have a dual effect; while they protect against certain actions of morphine they may, in other

respects, exhibit morphine-like activity themselves (Woods, 1956).

The relationship between the agonists causing the contraction of the longitudinal muscle coat, the antagonists morphine and the morphine inhibitors, nalorphine and levallorphan, is necessarily rather complex. As a full investigation was not intended at this stage, the single-dose technique was employed, namely, the action of a given dose of morphine on the action of a given amount of agonist was compared in the presence and absence of the morphine inhibitors.

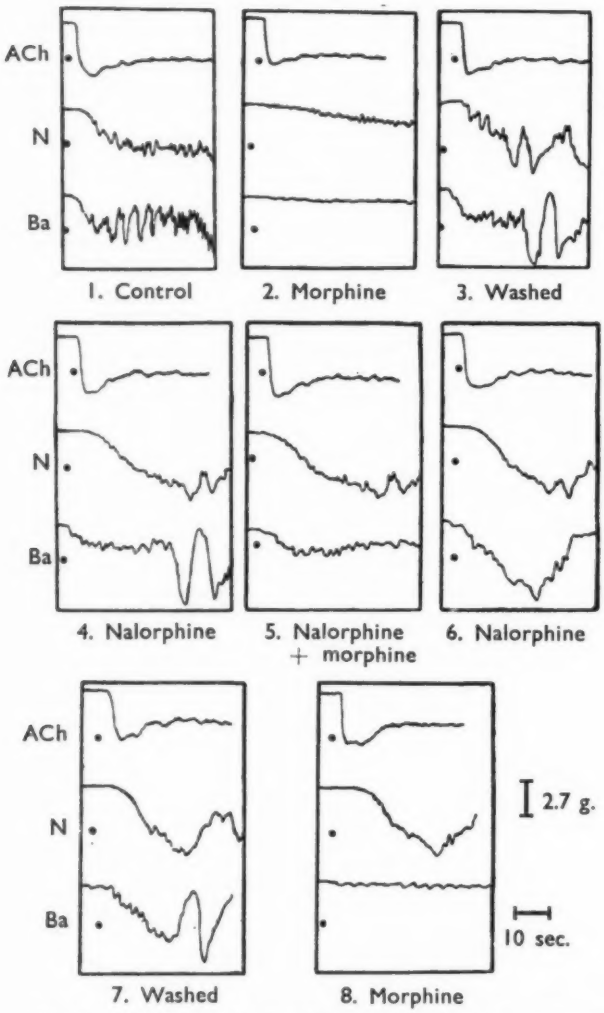

FIG. 7.—Inhibition of the morphine effect by nalorphine. Isometric contractions of the longitudinal muscle. Increase in tension downward. ACh, acetylcholine (0.0038 μg./ml.). N, nicotine (1.9 μg./ml.). Ba, barium (38 μg./ml.). Morphine (0.1 μg./ml.) was added 30 sec. before ACh, N or Ba. Nalorphine (0.075 μg./ml.) was left in the bath. 4, 12 to 18 min., 5, 27 to 36 min., 6, 48 to 54 min. after addition of nalorphine. 7, 27 to 36 min., 8, 39 to 48 min. after washing out nalorphine. The dots indicate the time when the drugs were added to the bath.

The assessment of the effects of the morphine inhibitors was made somewhat difficult by the considerable variation found between preparations. The general picture which emerged was that both nalorphine and levallorphan had a

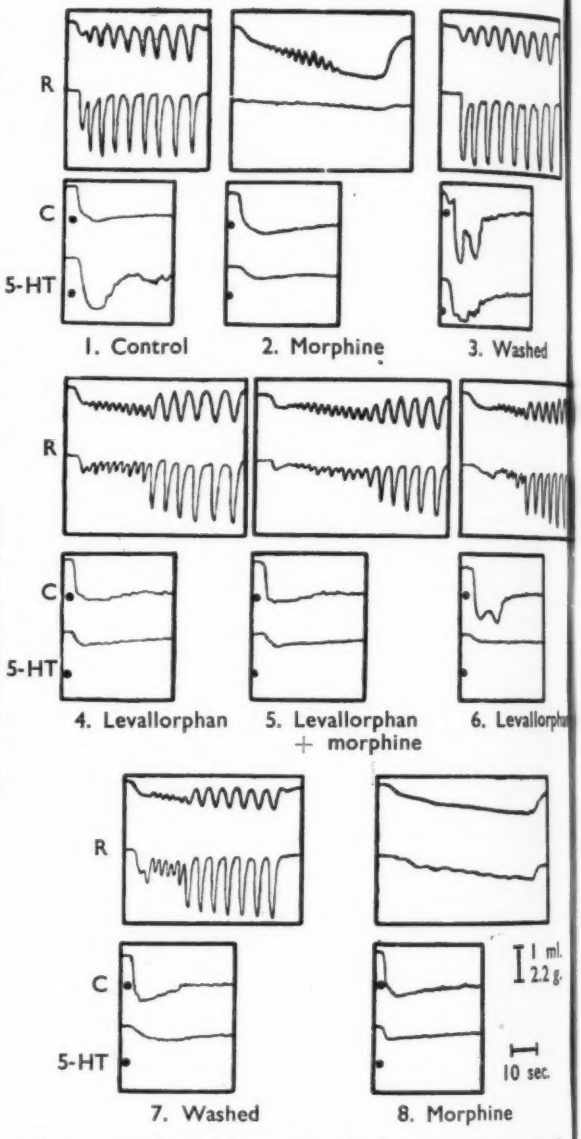

FIG. 8.—Inhibition of the morphine effect by levallorphan. Isometric contractions of the longitudinal muscle. Increase in tension or intestinal filling downward. R, peristaltic reflex after raising intra-intestinal pressure to 2.3 cm. H_2O. The upper tracing represents intra-intestinal volume changes and the lower tension changes in the longitudinal muscle. C, carbachol (0.0075 μg./ml.). 5-HT, 5-hydroxytryptamine (0.05 μg./ml.). Morphine (0.05 μg./ml.) was added 30 sec. before R, C, or 5-HT. Levallorphan (0.025 μg./ml.) was left in the bath. 4, 27 to 36 min., 5, 39 to 48 min., 6, 63 to 72 min. after addition of levallorphan. 7, 27 to 36 min., 8, 39 to 48 min. after washing out levallorphan. The dots indicate the time when the drugs were added to the bath.

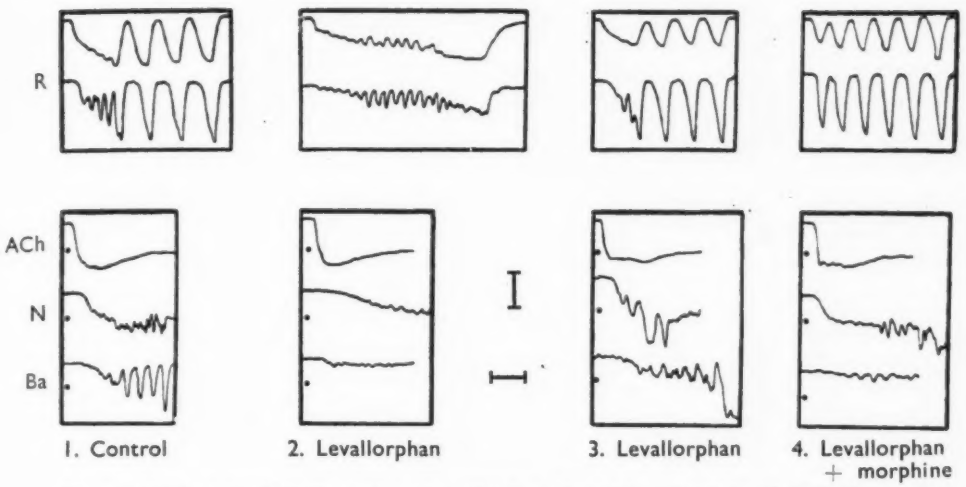

FIG. 9.—Transient morphine-like action of levallorphan. Isometric contractions of longitudinal muscle. Increase in tension or intestinal filling downward. R, peristaltic reflex after raising intra-intestinal pressure to 2 cm. H_2O. The upper tracing represents intra-intestinal volume changes and the lower tension changes in the longitudinal muscle. ACh, acetylcholine (0.006 μg./ml.). N, nicotine (2.5 μg./ml.). Ba, barium (50 μg./ml.). Morphine (0.05 μg./ml.) was added 30 sec. before R, ACh, N, or Ba. Levallorphan (0.05 μg./ml.) was left in the bath: 2, 9 to 18 min., 3, 27 to 39 min., 4, 42 to 51 min. after the addition of levallorphan. The dots indicate the time when the drugs were added to the bath. Vertical bar indicates 1 ml. or 3.6 g. Horizontal bar indicates 10 sec.

morphine-like and at the same time a morphine-inhibitory action and that the relative potencies of these actions varied with the agonists used. Thus, in the experiment shown in Fig. 7, nalorphine had no depressant effect on the contractions caused by nicotine and barium; it protected the nicotine contraction fully and the barium contraction partly against the effect of morphine. Half an hour after washing out the nalorphine, morphine scarcely affected the nicotine contraction but depressed the barium contraction.

In another experiment (Fig. 8) levallorphan partly depressed the peristaltic reflex and almost completely depressed the contraction caused by 5-HT. Morphine, in a concentration which in the absence of levallorphan abolished the peristaltic reflex, had only a small additional inhibitory effect on the reflex. The small residual contraction due to 5-HT was not further depressed by morphine. Half an hour after washing out the levallorphan, the peristaltic reflex was still partly inhibited and the 5-HT contraction showed only little recovery.

In some experiments, the depressant action of levallorphan was seen only during the first 10 to 20 min. after its addition to the bath; when the depressive phase had passed, the reflex and the nicotine contractions had become morphine-insensitive while the barium contraction was still inhibited by morphine (Fig. 9).

TABLE II

THE EFFECTS OF THE MORPHINE INHIBITORS, NALORPHINE AND LEVALLORPHAN, ON THE PERISTALTIC REFLEX AND THE CONTRACTIONS OF THE LONGITUDINAL MUSCLE CAUSED BY NICOTINE, BARIUM, AND 5-HYDROXYTRYPTAMINE

The numerals indicate numbers of experiments. Protection against morphine was tested when there was no or partial inhibition of the agonist by nalorphine or levallorphan; a 50% protection was considered positive. The number of experiments in which the morphine inhibitors produced a transient depression of the agonist are given in brackets. Doses: Morphine 0.025 μg./ml. Nalorphine 0.0125 to 0.0725 μg./ml. Levallorphan 0.0125 to 0.1 μg./ml.

Agonist	Nalorphine				Levallorphan			
	Antagonizes Agonist (Morphine-like Action)			Protects Agonist Against Morphine	Antagonizes Agonist (Morphine-like Action)			Protects Agonist Against Morphine
	Almost Completely	Partly	Not		Almost Completely	Partly	Not	
Intra-intestinal pressure (peristaltic reflex) ..	2	2	2 (1)	4	0	5	1 (1)	5
Nicotine	1	0	3	3	0	0	3 (1)	3
Barium	0	1	2	1	0	1	2 (1)	1
5-HT	2	0	0	—	5	0	0	—

The results of all experiments are summarized in Table II. Both nalorphine and levallorphan inhibited the 5-HT contractions in every preparation. The peristaltic reflex was at least partly depressed in 9 out of 12 experiments, showing a delayed onset of the peristaltic waves; on the other hand, the nicotine contractions were affected only occasionally. The modified reflex and the nicotine contractions were effectively protected against morphine. Although the barium contractions were depressed by the morphine inhibitors in only one third of the experiments, the protection against morphine was poor by either nalorphine or levallorphan.

Although morphine had very little effect on what was left of the 5-HT contractions after addition of either nalorphine or levallorphan (Fig. 8), this cannot at present be classed as a true protective effect. It is likely that this residual contraction is due to the action of 5-HT on the morphine-insensitive receptors.

Discussion

As the structure of the myenteric plexus and the relationship of most of its nerve cells to the smooth muscle fibres is by no means clear, the term "motor neurone" will be used to denote the nerve cells innervating the muscle fibres. It is felt that the term "post-ganglionic neurone" should be avoided since it is based on a preconceived idea of the anatomical arrangements in the myenteric plexus (Ambache, 1955).

In an analysis of the contraction of the longitudinal muscle coat during the preparatory phase of the peristaltic reflex it was found that this phase, which is not affected by ganglion blocking agents, is depressed by morphine, atropine, and lowering the bath temperature (Kosterlitz and Robinson, 1957). Therefore it was assumed that the reflex arc of the preparatory phase is probably non-synaptic.

Although an action of morphine on the afferent side of the reflex arc cannot be excluded, the evidence available so far suggests that at least most of its effects are on the efferent limb. There are obvious similarities between the contractions of the longitudinal muscle coat caused by intestinal distension on the one hand and those due to nicotine, barium, and 5-HT on the other. They are all depressed by lowering the bath temperature (Innes *et al.*, 1957) and by addition of morphine or atropine to the bath fluid. Further, botulinum toxin type D inhibits the action of nicotine, barium, and 5-HT (Ambache and Lessin, 1955).

The contractions of the longitudinal muscle layer caused by nicotine and barium are more complex than the contractions of the preparatory phase or that caused by 5-HT. When barium acts on fresh guinea-pig ileum, there is not only a sustained contraction of the longitudinal muscle but there are also contractions of a rhythmic type alternating with relaxations. This results in a pattern similar to that seen in the emptying phase of the peristaltic reflex and is often associated with a contraction wave of the circular muscle travelling in an aboral direction. Hexamethonium abolishes the oscillations of the longitudinal muscle and the contractions of the circular muscle but leaves the sustained contraction unchanged. It is assumed that the oscillations are caused by barium acting on cells of the intramural plexus involving hexamethonium-sensitive pathways to the "motor neurones," while the sustained morphine-sensitive contractions are due to a direct action on the "motor neurones." A similar analysis of the action of nicotine is impossible since hexamethonium seems to inhibit all nicotine receptors. 5-HT, which is not affected by hexamethonium, does not cause oscillatory contractions; the morphine-sensitive component of its action is believed to be on the "motor neurone."

Only part of the contractions caused by nicotine, barium, and 5-HT is inhibited by morphine and atropine. If it is assumed that the ratios of the doses which give identical contractions before and after the addition of a maximum dose of morphine are an indication of the susceptibility of the drug action to inhibition by morphine, then it follows that more than 80 to 85% of the original effects of nicotine and barium and over 95% of the original effect of 5-HT are inhibited by morphine. These figures are only very approximate as the shape of the dose/response curve is often altered by morphine. It should be noted that Gaddum and Picarelli (1957) found lower dose ratios for both 5-HT and nicotine; it is likely that the reason for this is to be found in different experimental conditions.

No definite evidence is available as to the nature of the morphine-resistant residual contractions. Gaddum and Picarelli (1957), investigating the inhibitory actions of morphine and phenoxybenzamine (dibenzyline) on contractions caused by 5-HT, concluded that the morphine-sensitive M receptors were probably in the nervous tissue and the morphine-resistant D receptors in muscle tissue. It therefore seems reasonable to assume that the morphine-resistant actions of nicotine and

barium are also on the muscle fibres themselves. This view is supported for barium, but not for nicotine, by the experiments of Ambache and Lessin (1955), who used botulinum toxin D to distinguish between action on neuronal and muscular receptors.

Once the actions of nicotine, barium, and 5-HT have been depressed by atropine, morphine has no further inhibitory effect. Conversely, atropine has only a small additive inhibitory effect on the morphine-treated ileum, a fact already observed for 5-HT by Gaddum and Picarelli (1957); only in the case of nicotine is this latter effect of some magnitude. When the special position of nicotine is disregarded, a likely explanation of the actions of morphine and atropine would be the assumption that both drugs act on different parts of the same neuro-effector unit. Since it has been shown that the amount of acetylcholine released after intestinal distension (Schaumann, 1956) or after electrical stimulation (Paton, 1956, 1957) is diminished by morphine, atropine seems to exert its effect more distally than morphine. This view has already been put forward by Gaddum and Picarelli (1957).

The fact that morphine inhibits the release of acetylcholine does not necessarily exclude the possibility of morphine interfering with the activity of the "motor neurone" at a site different from the point of acetylcholine release. This finds some support in the fact that the different agonists are affected in a different way by the morphine inhibitors. Thus, their morphine-like action decreases in the following order: 5-HT>intestinal distension>barium>nicotine and the degree of their morphine-inhibitory effect increases in approximately the same order: 5-HT<barium<intestinal distension<nicotine.

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THE STIMULATORY ACTION OF ACETYLCHOLINE ON ISOLATED RABBIT ATRIA

BY

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Quinidine (2.5 to 5×10^{-6} g./ml.) decreased the maximum rate of depolarization of the action potentials (AP) of both isolated rabbit atrial and ventricular fibres recorded with an intracellular microelectrode and finally abolished electrical excitability and spontaneous rhythmicity. Acetylcholine (ACh) 10^{-6} restored these properties in the atrium (but not ventricle) without changing the membrane resting potential (MRP). Adrenaline 10^{-6} to 10^{-4} at the same time was without effect. The maximum rate of depolarization of an AP in response to an extra shock interposed between the 8th and 9th driving stimuli was increased by ACh 10^{-6} in atria which had received quinidine to a greater extent than that of the normal AP. It is suggested that ACh restarted atria arrested by quinidine by an action on the sodium "carrying system" (excitatory mechanism) and this was not the result of an increase in MRP. An hypothesis is put forward to explain the stimulatory and inhibitory effects of ACh on rabbit atria.

Acetylcholine (ACh) restarts isolated rabbit atria which have been arrested by quinidine (Briscoe and Burn, 1954). Armitage (1957) suggested that quinidine decreases the potassium permeability of the cell membrane so that repolarization becomes less and less complete and that ACh restarts such atria by increasing potassium permeability. This obviously implies a concomitant restoration of the membrane resting potential (MRP).

However, this hypothesis has not been substantiated with electrophysiological evidence and the results do not exclude the obvious alternative that the interaction of quinidine and ACh is on the excitatory mechanism (Johnson and Robertson, 1957). Indeed this latter possibility is compatible with the findings of Weidmann (1955a), Johnson (1956) and Johnson and McKinnon (1957a) that the action of quinidine on other cardiac tissue is directly on the excitatory mechanism, for it predominantly affected the depolarization phase of the action potential without changing the MRP.

The experiments in the present paper were designed to show whether there was any change in MRP associated with the restarting of quinidinized atria by ACh and whether there was any evidence to suggest that ACh itself had an effect on the sodium carrying system, namely the excitatory mechanism (Hodgkin and Huxley, 1952; Weidman, 1955b).

METHODS

The apparatus used was basically similar to that described previously (Johnson, 1956). The salient features of the floating grid electrometer cathode follower input stage (Murray, 1955, personal communication) without microelectrode were: (1) the rise time was <1 μsec., (2) the input resistance was $>10^{16}$ ohms, (3) the input capacitance was $<10^{-2}$ $\mu\mu$F., (4) the current drawn from cell by the microelectrode was $<10^{-16}$ A., (5) the grid current with ± 10 volt grid swing was $<10^{-15}$ A. and the drift ± 2 mV/hr.

Important modifications and differences in apparatus and technique from those described previously are as follows: (1) The organ bath was redesigned with an improved temperature stabilization (better than $\pm$ 0.1°) and was equipped with a temperature measuring device of low heat capacity and rapid response time, which was sensitive to changes of temperature of the muscle of 0.01°. (2) A feedback differentiator was modified to be capable of registering rates of rise from 1.5 to 1,500 V./sec. (3) The microelectrodes were filled by boiling in 3 M-KCl under reduced pressure for 20 min.

Isolated rabbit right atria or ventricle in Krebs-Henseleit solution were used. The tissue was driven at a constant rate of approximately 150/min. using external electrodes. In some experiments an extra shock was interposed at variable times between the

8th and 9th driving stimuli. The "single cell technique" was used (Johnson and McKinnon, 1957a) by which any one set of observations was made from recordings of the one cell.

The drugs used were quinidine sulphate (Burroughs Wellcome), acetylcholine chloride (Roche), and hyoscyamine sulphate.

They were made up in Krebs-Henseleit, added directly to the organ bath and washed out by passing fresh Krebs-Henseleit solution through the bath.

Results

Effect of ACh on the Membrane Resting Potential of Atria Arrested by Quinidine

The effects of ACh (10^{-6} g./ml.) on normal atria were identical to those described for isolated guinea-pig atrium (Johnson and McKinnon, 1957b), consisting of an increase in the rate of repolarization with no change in membrane resting potential (MRP), maximum rate of depolarization or amplitude of the action potential. When the tissue was exposed to quinidine 2.5 to 5×10^{-6}, the typical effects already described for Purkinje and ventricular fibres (Weidmann, 1955a; Johnson, 1956) were seen, namely a progressive decrease in the maximum rate of depolarization which was accompanied eventually by a fall in the amplitude of the action potential, and finally, after a period of approximately 10 to 20 min., the atrium failed to respond to electrical stimuli and lost its spontaneous rhythmicity. At this point ACh 10^{-6} was applied and in every case (10/10) there was a transient appearance of propagated action potentials of low voltage and low rate of depolarization. The maximum rate of depolarization and amplitude of these action potentials rose and fell during the period of their transient appearance (10 to 60 sec.). This restoration of electrical excitability produced by ACh was never accompanied by any detectable change in MRP. Further applications of ACh frequently restored electrical excitability for several minutes and also restored spontaneous rhythmicity. All these effects were abolished or prevented by hyoscyamine (10^{-6}). This "stimulating" action of ACh appears identical to that described by Briscoe and Burn (1954). In view of the failure to detect any change in the MRP, it appeared worth while to proceed with an investigation of the effects of ACh on the "sodium carrying system."

Effect of ACh on the "Sodium Carrying System" of Normal and Quinidinized Atria

The maximum rate of depolarization can be considered as proportional to the sodium current into the cell or the activity of the "sodium carrying system" (Hodgkin and Katz, 1949; Weidmann, 1955b). By observing the change in the maximum rate of depolarization of an action potential due to an extra shock interposed between the 8th and 9th driving stimuli, an index can be obtained of the effects of a drug on the rate of recovery of the "sodium carrying system."

In the normal fibre, it was frequently impossible to initiate an extra action potential of diminished rate of rise compared with the normal without evoking it during the latter part of the repolarization phase of the previous action potential (see Fig. 1*a*). This indicates that the rate of recovery

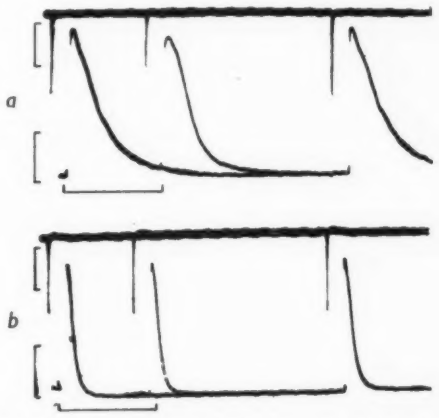

FIG. 1.—Effect of ACh on the action potential (AP) of the normal isolated rabbit atrium driven at 210/min. In both (*a*) and (*b*): upper trace, first differential of AP; lower trace, AP. In each trace, the first and last AP are in response to the 8th and 9th driving stimuli and the middle AP to the extra shock. Calibration: Upper ordinate, 100 V./sec.; lower, 30 mV.; abscissa, 100 msec. (*a*) Shows extra AP of diminished maximum rate of depolarization (1st differential) evoked during the repolarization phase of previous AP. (*b*) After 30 sec. exposure to ACh 10^{-6}. The maximum rate of depolarization of the extra AP has increased equalling those of the normal AP.

of the "sodium carrying system" of the normal fibre closely followed the repolarization phase of the action potential.

The application of ACh 10^{-6} increased the maximum rate of depolarization of the extra shock under these circumstances often to that of the "normal" action potential (see Fig. 1*b*). This is most likely due not to a direct effect on the rate of recovery of the "sodium carrying system" but to the accompanying increase in the rate of repolarization produced by ACh, since, after ACh, the extra action potential was now evoked at a time when the membrane potential had returned to normal.

If after ACh an extra action potential was evoked, especially during the latter part of the repolarization phase, repetitive firing in response

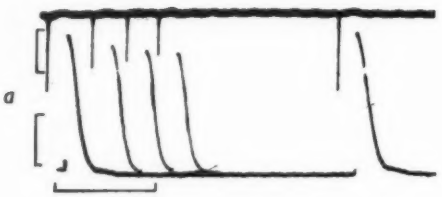

FIG. 2.—Effect of ACh on the action potential (AP) of the normal isolated rabbit atrium driven at 210/min. Same cell as in Fig. 1. In both (*a*) and (*b*): Upper trace, first differential of the AP; lower trace, AP. Calibration: upper ordinate, 100 V./sec.; lower, 30 mV.; abscissa, 100 msec. (*a*) After 60 sec. exposure to ACh 10^{-6}. The first and last AP are in response to the 8th and 9th driving stimuli. The second AP is in direct response to an extra shock; note repetitive firing following this extra AP. (*b*) After 75 sec. exposure to ACh 10^{-6} sustained repetitive firing originating as in (*a*). Record is of two consecutive traces superimposed with free running time base.

to this extra shock was often observed (see Fig. 2) (Hoffman and Suckling, 1953). Suggestion of a direct effect of ACh on the excitatory mechanism was afforded by the occasional observation that, in the untreated fibre with an unusually low rate of depolarization, ACh 10^{-6} increased the maximum rate of rise and amplitude of the action potentials (in response to the normal driving stimuli) with no detectable change in MRP.

During the early stages of treatment with quinidine, it was frequently possible to evoke, prior to arrest, an action potential of diminished rate of depolarization at a time when repolarization of the previous action potential was complete (see Fig. 3*a*). This indicates that quinidine can produce a dissociation between repolarization and restoration of the "sodium carrying system" as suggested previously by Weidmann (1955a) and Johnson and McKinnon (1957a). Application of ACh 10^{-6} under these conditions increased the maximum rate of depolarization of both the normal and extra action potentials. The latter was affected to a greater extent and frequently the two values became similar (see Fig. 3*b*).

It appears that ACh increases the maximum rate of depolarization of the extra and the normal action potentials by an effect on the "sodium carrying system" which is not the result of a change in MRP; for the extra action potential was evoked some time after repolarization of the previous action potential and ACh had no effect on the MRP.

Effect of Adrenaline in Atria Arrested by Quinidine

In the only experiment in which it was tested, adrenaline failed to restore electrical excitability of the atria arrested by quinidine 5×10^{-6}. However, the presence of adrenaline did not affect the restoration of electrical excitability by ACh 10^{-6} added immediately afterwards. This phenomenon was repeated several times.

Therefore it appears that the restarting action of ACh is not due to its causing the release of adrenaline nor does adrenaline affect this stimulatory action of ACh.

Effect of ACh in Normal and Quinidinized Ventricle

ACh 10^{-6} to 10^{-4} did not restore in 3/3 experiments the electrical excitability of isolated rabbit ventricle which had been arrested by cooling or quinidine. ACh had no effect on the maximum rate of depolarization of partially quinidinized

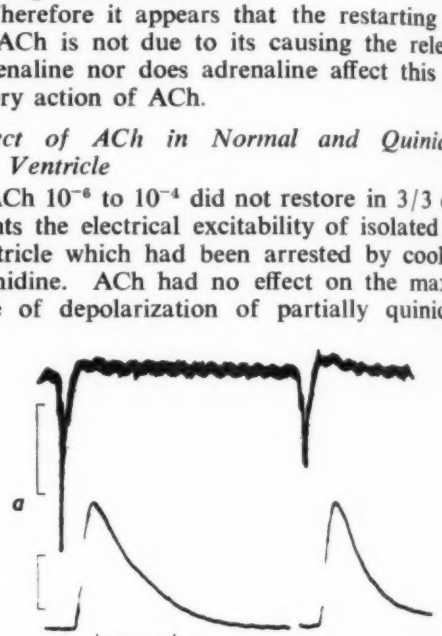

FIG. 3.—Effects of ACh on the action potential (AP) of the isolated rabbit atrium driven at 165/min. in the presence of quinidine. In both (*a*) and (*b*): Upper trace, first differential of AP; lower trace, AP. In each trace, the first AP is in response to the 8th driving stimulus and the second to an extra shock. The tissue did not respond to the 9th driving stimulus, for it fell in the refractory period of the extra AP. Calibration: upper ordinate 7.5 V./sec.; lower, 30 mV.; abscissa, 100 msec. (*a*) 10 min. exposure to quinidine 5×10^{-6}. Note that, although the extra AP is evoked some time after repolarization of the preceding "normal" AP is completed, its maximum rate of depolarization (1st differential) is less than that of the latter. (*b*) as in (*a*) but after 30 sec. exposure to ACh 10^{-6}. Both maximum rates of depolarization are increased, that of the extra AP more than the "normal."

ventricle or on that of an extra action potential evoked between the 8th and 9th driving stimuli in normal and quinidinized ventricle.

Discussion

The results of the present work clearly indicate that ACh can restart an atrium arrested by quinidine without raising the MRP. They show that ACh has a restorative effect on the "sodium carrying system." Previous work has shown that quinidine interferes with this system (Weidmann, 1955a: Johnson and McKinnon, 1957a) and this interference is sufficient to explain the ultimate arresting action of quinidine in rabbit atrium.

The hypothesis proposed by Armitage (1957) and supported by Burn (1956) that ACh restarts an atrium arrested by quinidine by increasing the potassium permeability of the cell membrane thus presumably restoring the MRP is not supported by our findings. Their hypothesis is based on the fact that by reducing the potassium concentration in the bathing medium (K_o) the rate and size of contractions of atria which have been depressed by quinidine were increased again. The most probable explanation for this effect is that the reduction in K_o to $\frac{1}{4}$ and $\frac{1}{2}$ normal would result in an increase in MRP. Judging from the results of Weidmann (1955b) this hyperpolarization would cause an increased availability of the "sodium carrying system" giving an increase in the number of resting sodium-carrying units and an increase in the maximum rate of depolarization of a subsequently evoked action potential.

We have recently obtained further evidence of an action of ACh on the excitatory mechanism of rabbit atrial cells, for, even though it reduces the membrane resistance, it decreases the amount of depolarization required to initiate an action potential (Johnson and Robertson, 1958). Marshall (1957) has shown that cooling causes a fall in the MRP of rabbit atrial fibres and the restarting action of ACh in these circumstances is accompanied by a rise in membrane potential. However, ACh can restart isolated rabbit atrial fibres arrested by cooling without altering the MRP (Johnson and Robertson, unpublished observations). Thus it appears that the excitatory actions of ACh on the heart described by Burn (1956) cannot be attributed entirely to ACh increasing the MRP and the rate of repolarization.

We reaffirm the following hypothesis (Johnson and Robertson, 1957) which takes into account the action of ACh on the rate of repolarization, the membrane potential and the excitatory mechanism. The usual inhibitory action of ACh on the rate of beat of pacemaker cells is probably due, as is well known, to an increase in potassium permeability which diminishes the rate of diastolic depolarization responsible for rhythmicity. The arrest of atria by quinidine, cooling and fatigue and the restarting by ACh are almost certainly the result of inactivation and restoration of the "sodium carrying system." Although the availability of this system is directly related to the membrane potential (Weidmann, 1955b), it can also be affected independently of it as in the case of quinidine. One or both of these two mechanisms are involved in the restoration by ACh of the availability of this system in arrested atria. Thus the effect of ACh on the MRP may be the more important in the case of fatigued fibres which are reported to have a low MRP (Weidmann, 1955a). On the other hand ACh can restart quinidinized atria by the more direct effect on the "carrying system" and finally both mechanisms could operate in the case of cooled fibres when a change in MRP occurs.

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PHARMACOLOGICAL PROPERTIES OF ββ-DIMETHYLACRYLOYLCHOLINE AND SOME OTHER β-SUBSTITUTED ACRYLOYLCHOLINES

BY

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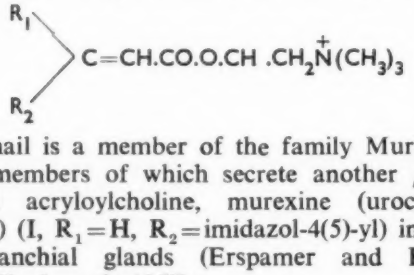
The mammalian pharmacology of a new naturally occurring ester of choline, ββ-dimethylacryloylcholine (DMAC), has been studied, mainly in the cat, together with that of two synthetic β-substituted acryloylcholines, crotonoylcholine and pent-2-enoylcholine. Comparisons have been made with the reduced form of DMAC, *iso*valerylcholine, with another naturally occurring β-substituted acryloylcholine, murexine (urocanoylcholine), and with suxamethonium. DMAC has been shown to be a ganglion stimulating and neuromuscular blocking agent generally similar, in potency and properties, to murexine. It is also a powerful respiratory stimulant. The other unsaturated esters behaved similarly but were less potent.

ββ-Dimethylacryloylcholine (DMAC, in I, $R_1 = R_2 = CH_3$) has recently been added to the growing number of pharmacologically active esters of choline known to occur in nature (Whittaker, 1957; Keyl, Michaelson, and Whittaker, 1957). It is present in relatively high concentration in the hypobranchial gland of the marine prosobranch gastropod, *Thais floridana* (Southern oyster drill).



This snail is a member of the family Muricidae, other members of which secrete another β-substituted acryloylcholine, murexine (urocanoylcholine) (I, $R_1 = H$, R_2 = imidazol-4(5)-yl) in their hypobranchial glands (Erspamer and Benati, 1953; Keyl *et al.*, 1957).

The mammalian pharmacology of DMAC has now been studied. Two other new β-substituted acryloylcholines, crotonoylcholine (I, $R_1 = H$, $R_2 = CH_3$) and pent-2-enoylcholine (I, $R_1 = H$, $R_2 = CH_3.CH_2-$), have been included for comparison, as have *iso*valerylcholine (the reduced form of DMAC), murexine and suxamethonium. Of these, only murexine occurs in nature, so far as is known. The new ester has been found to resemble murexine (previously studied *inter alia* by Erspamer and Glässer, 1957, and Keyl and Whittaker, 1958) in being a moderately effective neuromuscular blocking agent of the depolarizing type and in having ganglion-stimulating properties. It is also a powerful respiratory stimulant. With murexine, the last two effects are more in evidence in the dog than in the cat, the species mainly studied in the present work. The other new substituted acryloylcholines behaved similarly but were less effective.

MATERIALS AND METHODS

Compounds Used.—Acetylcholine, DMAC, crotonoylcholine, pent-2-enoylcholine, *iso*valerylcholine and suxamethonium (all as iodides, unless otherwise stated) were kindly placed at our disposal by Dr. L.-E. Tammelin, Research Institute of National Defence, Sundbyberg, 4. Murexine (urocanoylcholine) chloride hydrochloride was kindly provided by Professor V. Erspamer. Other compounds used were: dibenamine hydrochloride (Lilly); dihydroergotamine (Sandoz); hexamethonium bromide (Vegolysen, May and Baker); allobarbitone (Dial); eserine salicylate; atropine sulphate (Swedish pharmacopoeia, XIth ed.).

Recording of Respiration, Blood Pressure, and Muscular Contractions.—The effect of DMAC and the other choline esters was studied in over 20 anaesthetized cats. Anaesthesia was induced with allobarbitone (50 mg./kg. body weight) and was maintained by further small doses of allobarbitone. Drugs were injected intravenously through a plastic

cannula in the femoral vein. Respiration, blood pressure, and contractions of the gastrocnemius muscle and of the nictitating membrane were recorded by passing the output of suitably arranged transducers into a Grass Model 3 D electroencephalograph.

Respiration was recorded as pressure differences in the tracheal cannula by means of a pressure transducer (Model PT 5, Grass Instrument Corporation). Blood pressure was recorded by means of a Statham Electromanometer from the left carotid artery. The effect of the drugs on neuromuscular transmission was determined by recording isometric contractions of the gastrocnemius muscles in response to supramaximal electrical stimulation of the sciatic nerve. A force-displacement transducer Model FT 10, Grass Instrument Corporation, was used for recording the muscle contractions and the electrical stimuli were applied to the nerve distally to a crushed region by means of a Grass stimulator, Model S 4, and shielded silver electrodes. The neuromuscular blocking effect of the drugs is expressed in terms of the PD50 (namely, the dose required to produce 50% blockade) and of the duration of such a block. To determine these quantities, the drugs were injected intra-arterially through a cannula in the femoral artery of the leg opposite to that being stimulated, the tip of the cannula lying proximally to the aortic bifurcation. The percentage diminution of twitch height and duration of block were recorded for 3 doses and the PD50 and the corresponding duration estimated by graphical interpolation.

Contractions of the nictitating membrane were recorded by means of force-displacement transducer FT 03. The cervical sympathetic trunk was exposed and dissected from the vagus. The excitability of the membrane was tested by stimulating the preganglionic trunk for 5 sec. with rectangular pulses of duration of 10 sec., amplitude of 150 mA., and at a frequency of 50 cycles/sec. before and after administration of DMAC and hexamethonium.

Denervation of the Carotid Sinus.—The sinus nerves were freed bilaterally by careful dissection, stimulated to elicit a depressor response, then cut, and the internal carotid artery dissected free, ligated and cut. The vagi were cut on both sides.

Functional Adrenalectomy.—The adrenals were exposed retroperitoneally through lateral incisions on both sides, their blood supply was clamped off by suitable forceps and the experiment continued without removal of the adrenals.

Blood Flow Measurements.—Blood flow was recorded by the method of Lindgren (1958). All injections were given in saline intra-arterially in a volume of 0.1 ml. Acetylcholine was used as a standard.

Frog Rectus Abdominis Muscle Preparations.—The procedure was essentially that of Chang and Gaddum (1933). In this and the following preparation the activity of the esters was expressed as the relative molar potency in percentage units, namely, as the number of moles of acetylcholine giving a response equivalent to 100 moles of active substance at the dose levels compared.

Guinea-pig Ileum Preparations.—A test bath of 6 ml. volume was used. An automatic timer regulated the cycle of operations except for the addition of the compounds which was made manually in a small volume on top of the test bath.

Hydrolysis of Esters by Cholinesterases.—This was measured manometrically at 37° and *p*H 7.4 in 0.023M-$NaHCO_3$ in equilibrium with 5% v/v CO_2 in N_2 in the gas phase (Ammon, 1933) or electrometrically at 25° and *p*H 8 in barbitone buffer as described by Tammelin (1953). The following preparations were used: purified bovine red cell cholinesterase (Winthrop-Stearns Inc.); freeze-dried electric organ from *Torpedo ocellata*; purified human plasma cholinesterase stated to correspond to Cohn's plasma fraction IV-6-3 (AB Kabi). The rate of hydrolysis was expressed in terms of the Q value, namely, μl. acid evolved/mg. enzyme preparation/hr. for acetylcholine or as % of the acetylcholine rate for the other esters. The substrate concentrations, unless otherwise stated, were: acetylcholine, 30 mM (human plasma cholinesterase) or 10 mM (other cholinesterases); other esters, 10 mM. Enzyme concentrations were adjusted to give convenient rates of hydrolysis with the different substrates.

Results

Animal Experiments

Effect on Respiration.—All three unsaturated choline esters stimulated respiration; DMAC was the most powerful respiratory stimulant of the three and evoked a marked response at a dose of 0.1 mg./kg. (Figs. 1 to 6). The effect was noticed immediately upon injection of the compound and did not seem to bear any direct relationship to the neuromuscular blockade. When a biphasic effect on blood pressure was evident, the stimulation of respiration coincided with the first, rising phase (Fig. 2*a*). The animals developed a forced respiration and an increased respiratory rate which lasted less than 1 min. The effect then subsided and in some cases was succeeded by an apnoea due to the hyperventilation.

The respiratory effect of DMAC was moderately potentiated by eserine, somewhat reduced by atropine (Fig. 1), and considerably reduced by the ganglionic blocking agent hexamethonium (Fig. 5). The anti-adrenaline compound dibenamine did not greatly influence the primary respiratory response to the drug, but induced a secondary response which was probably a consequence of the altered effect of the drug on the blood pressure. This can be seen in Fig. 4, where it will be observed that the prolonged fall of blood pressure caused

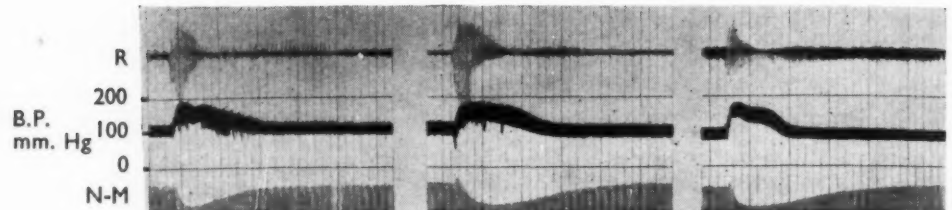


FIG. 1.—Effect of DMAC on respiration (R), blood pressure (B.P.) and neuromuscular transmission (N-M). Cat, 2·1 kg. Anaesthetic, allobarbitone. Time, 10 sec. D = Injection of DMAC (0·25 mg./kg.). E = Injection of eserine salicylate (0·15 mg./kg.). A = Injection of atropine sulphate (2·5 mg./kg.).

by DMAC and *iso*valerylcholine after administration of dibenamine was associated with a more long-lasting hyperpnoea distinct from the primary stimulant effect exerted by DMAC alone.

To find out if the stimulation of the respiration was produced centrally or by way of the chemoreceptors of the carotid sinus, DMAC and *iso*valerylcholine were compared before and after complete denervation of the sinus and bilateral vagotomy. Denervation of the sinus completely abolished the respiratory stimulant effect of DMAC without significantly affecting the blood pressure and the neuromuscular transmission (Fig. 2). It is therefore concluded that the effect of DMAC is brought about by stimulation of the chemoreceptors of the carotid sinus. The effect of eserine and atropine on the response to DMAC is consistent with what is known about the pharmacology of the carotid sinus (Heymans, 1955).

Clamping off the adrenals had no measurable influence on the respiratory stimulation brought about by DMAC (Fig. 3*b*), showing that the initial respiratory stimulation is not due to a release of pressor amines from the adrenals.

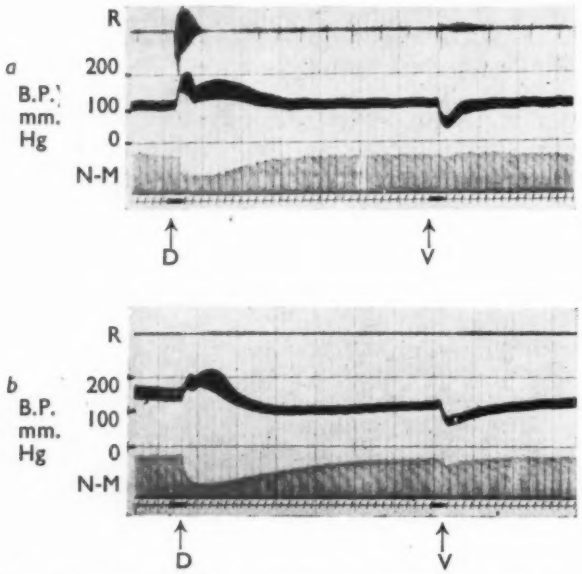


FIG. 2.—Effect of DMAC and *iso*valerylcholine on respiration, blood pressure and neuromuscular transmission. Cat, 2·5 kg. Anaesthetic, allobarbitone. Time, 10 sec. *a* before, *b* after denervation of sinus and cutting of vagi. D = Injection of DMAC (0·1 mg./kg.). V = Injection of *iso*valerylcholine (0·1 mg./kg.).

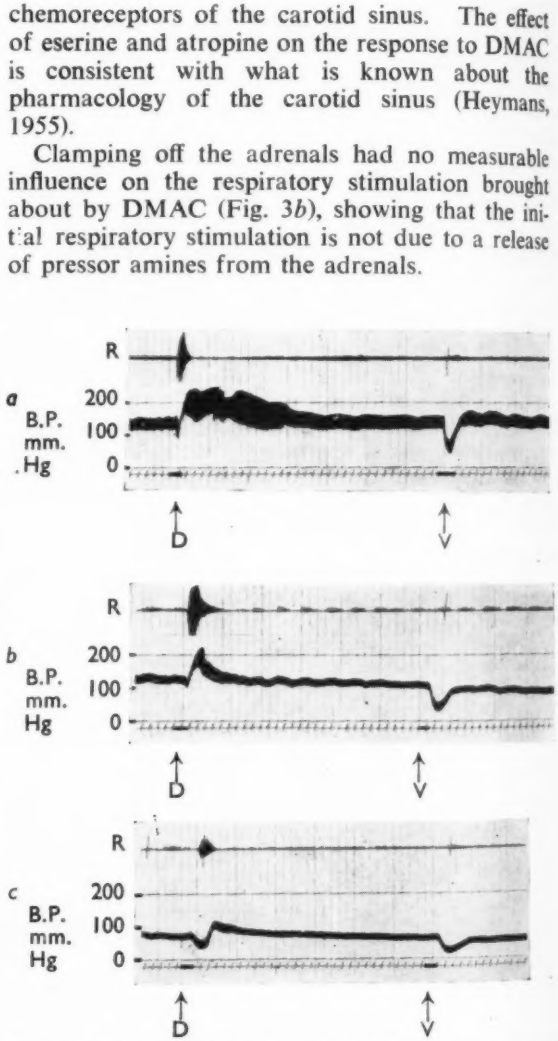


FIG. 3.—Effect of DMAC and *iso*valerylcholine on respiration and blood pressure. Cat, 3·4 kg. Anaesthetic, allobarbitone Time, 10 sec. *a* before, *b* after clamping off blood supply to adrenals, *c* after injection of 1 mg. dihydroergotamine. D = Injection of DMAC (0·1 mg./kg.). V = Injection of *iso*valerylcholine (0·1 mg./kg.).

Effect on Blood Pressure and Nictitating Membrane.—DMAC produces a rise in blood pressure when given intravenously to cats (Figs. 1 to 5). By contrast, pent-2-enoylcholine and crotonoylcholine (in doses of 0.1 to 0.2 mg./kg.) produce a rise in blood pressure only after eserinization and this pressor effect is further potentiated by atropine (Fig. 6), while *iso*valerylcholine has only a depressor effect (Figs. 2 to 4).

When DMAC was injected intravenously, a biphasic effect was sometimes noted (Figs. 2*a*, 3*a*) consisting of an initial rapid rise in blood pressure and then a more sustained pressor effect which gradually wore off. Sinus denervation affected neither of these phases, although abolishing the respiratory stimulant effect completely. When the adrenals were clamped off, only the rapid increase in blood pressure remained (Fig. 3*b*). Dibenamine completely reversed both pressor effects (Fig. 4), as did dihydroergotamine (Fig. 3*c*). Ganglionic blockade by hexamethonium abolished the pressor effect and caused no reversal (Fig. 5). Eserinization or atropinization had very little, if any, effect upon the blood pressure.

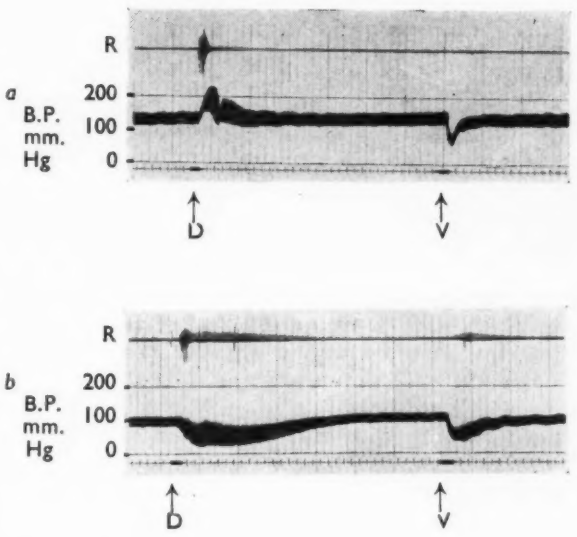


FIG. 4.—Effect of DMAC and *iso*valerylcholine on respiration and blood pressure. Cat, 3 kg. Anaesthetic, allobarbitone. Time, 10 sec. ***a*** before, ***b*** 45 min. after the injection of dibenamine hydrochloride (15 mg./kg.). D = Injection of DMAC (0·1 mg./kg.). V = Injection of *iso*valerylcholine (0·1 mg./kg.).

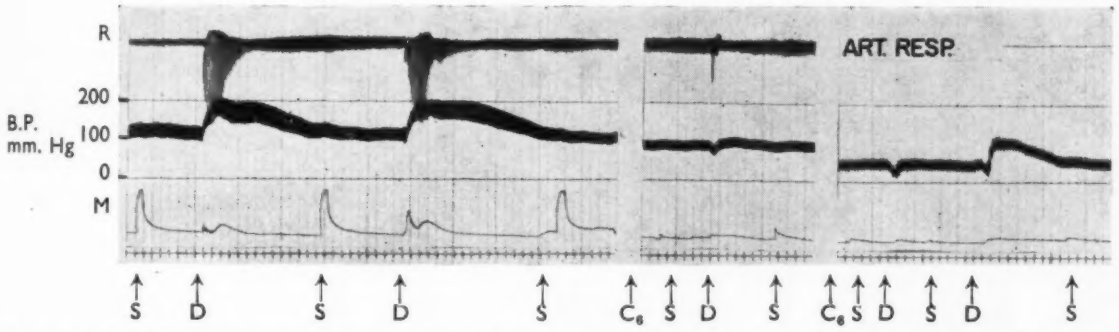


FIG. 5.—Effect of DMAC on respiration, blood pressure and nictitating membrane (M). Cat, 4·5 kg. Anaesthetic, allobarbitone. Time, 10 sec. D = Injection of DMAC; the first injection, 0·1 mg./kg.; the last injection, 1 mg./kg.; the other injections, 0·2 mg./kg. C_6 = Injection of hexamethonium bromide (5 mg./kg.). S = Stimulus of isolated central stump of cat cervical sympathetic trunk for 5 sec. with rectangular pulses of 10 msec. duration at a frequency of 50 c./sec. and intensity of 150 mA.

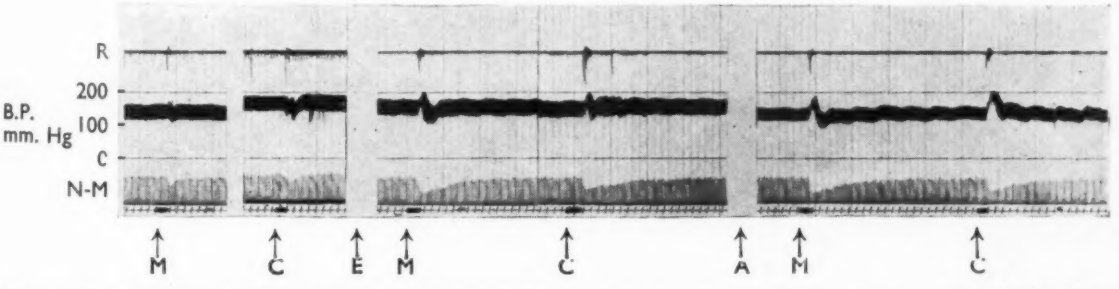


FIG. 6.—Effect of crotonoylcholine and pent-2-enoylcholine on respiration, blood pressure and neuromuscular transmission. Cat, 4·7 kg. Anaesthetic, allobarbitone. Time, 10 sec. M = Injection of pent-2-enoylcholine (0·2 mg./kg.). C = Injection of crotonoylcholine (0·1 mg. kg.). E = Injection of eserine salicylate (0·1 mg./kg.). A = Injection of atropine sulphate (1 mg./kg.).

From these results it is concluded that the first component of the pressor response is caused by sympathetic ganglionic discharge and the second by released pressor amines. That DMAC stimulates the sympathetic ganglia is evinced by Fig. 5, where the effect on the tone of the nictitating membrane of stimulation of the cervical sympathetic and injections of DMAC have been recorded. It will be seen that increasing doses produced increases in tone of the membrane, and both this effect and the effect of stimulation are abolished by the ganglionic blocking agent, hexamethonium. It will also be noted that the stimulation of the superior cervical ganglion by DMAC coincides with the first rapid phase of the pressor effect and also with the period of stimulation of the respiration. It is known that ganglionic stimulation and stimulation of the carotid sinus often run parallel (Heymans, 1955).

Effect on Neuromuscular Transmission.—All three of the new β-substituted acryloylcholines showed neuromuscular blocking activity (Figs. 1, 2, 6, and 7). It will be seen from Fig. 7 that DMAC has about 70% of the potency of murexine

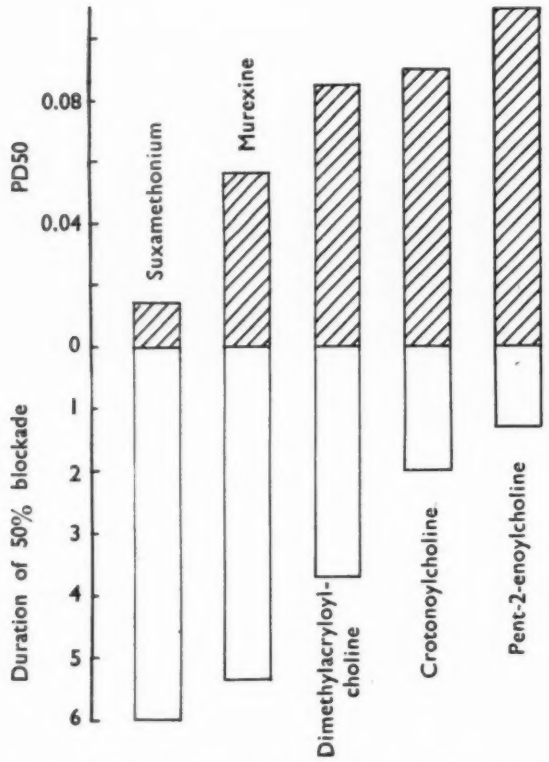


FIG. 7.—PD50 (mg./kg., intra-arterially) and duration of 50% blockade (min.) produced by a series of β-substituted acryloylcholines in the cat. Suxamethonium is included for comparison.

on the basis of either the PD50 or the duration of 50% blockade; crotonoylcholine and pent-2-enoylcholine are less effective than DMAC, in that order. All the β-substituted acryloylcholines are less effective than suxamethonium. The blocking effect of the new compounds was intensified by eserine (Figs. 1 and 6), suggesting that the block was primarily of the depolarizing type.

Activity of Esters on Frog Rectus and Guinea-pig Ileum.—The activities of the three acryloylcholines and *iso*valerylcholine on these preparations are given in Table I. The relative molar potencies of the esters on the frog rectus showed considerable variations in different preparations; the values given in Table I are representative of the present series of results, but are, with one exception, higher than those recorded earlier (Keyl *et al.*, 1957). It will be noted that all the esters are quite active, but none more so than acetylcholine.

TABLE I

ACTIVITY OF β-SUBSTITUTED ACRYLOYLCHOLINES ON FROG RECTUS ABDOMINIS MUSCLE AND GUINEA-PIG ILEUM

Ester	Relative Molar Potency (Acetylcholine = 100)	
	Frog Rectus	Guinea-pig Ileum
DMAC	70	0·13
Crotonoylcholine	16	0·14
Pent-2-enoylcholine	45	1·3
*iso*Valerylcholine	57	0·02

By contrast, the esters showed only very feeble activity on the guinea-pig ileum. Not only were the doses which were needed to elicit a contraction very high, but the response of the muscle differed from that obtained with acetylcholine in that the initial phase of rapid contraction was succeeded by a phase of fluctuating and gradually decreasing tone and maximum contractions could not be elicited. This suggests that the esters were acting by way of the ganglia rather than on the muscle directly.

Effect on Peripheral Blood Flow.—When compared with acetylcholine, which has a well-known peripheral vasodilator effect (Folkow and Uvnäs, 1948; Folkow, Frost, Haeger, and Uvnäs, 1949), the unsaturated esters show a very weak and non-specific activity. DMAC is 1/100th to 1/10th less active than acetylcholine (Fig. 8), but, unlike the latter, increasing doses do not produce increased vasodilatation; indeed, the response if anything decreases (see Fig. 8, injections 7 and 8). Pent-2-enoylcholine seems to be totally without action on

the muscular blood flow in the dose range tested (see Fig. 9, injections 6 to 9), while crotonoylcholine produced a very slight response (see Fig. 9, injections 10 to 13).

In conclusion, the unsaturated choline esters when injected intra-arterially have a very weak vasodilator effect on the muscular blood vessels; this effect is presumably peripheral, since no concomitant changes in blood pressure were observed (Figs. 8 and 9). This is consistent with their feeble effect on the guinea-pig ileum, and in conjunction with the frog rectus and blood pressure results indicates that DMAC and the other unsaturated esters have ganglion stimulating (" nicotine-like ") but no smooth muscle stimulating (" muscarine-like ") activity.

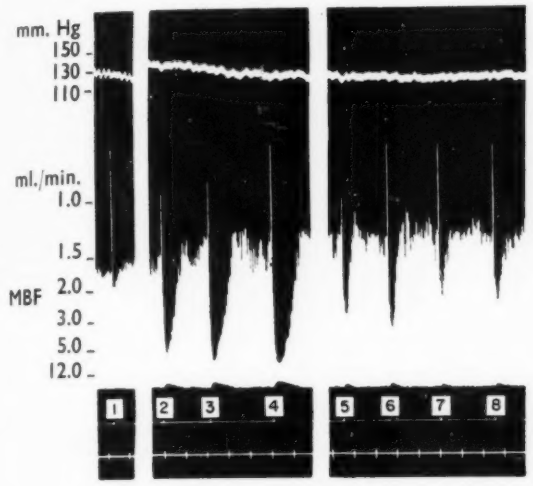


FIG. 8.—Effect of acetylcholine and DMAC bromide on blood pressure and muscular blood flow (MBF) in the right hind limb recorded in the femoral artery. Cat, 2·8 kg. Anaesthetic, allobarbitone. Time, min. The following injections were made intra-arterially in 0·1 ml. saline. 1, saline alone; 2 to 4, acetylcholine (2, 0·001 μg.; 3, 0·1 μg.; 4, 0·1 μg.); 5 to 8, DMAC bromide (5, 6, 0·1 μg.; 7, 8, 1 μg.).

TABLE II

HYDROLYSIS OF β-SUBSTITUTED ACRYLOYLCHOLINES BY CHOLINESTERASES

(a) = determined manometrically, (b) = determined electrometrically, (c) = the same result was obtained electrometrically with 30 mM DMAC and (d) this estimate was based on the assumption that the preparation contained 3% enzyme preparation in stabilizer as stated by the manufacturer.

Preparation	Acetylcholine (Q)	DMAC	Croton-oyl choline	Pent-2-enoyl-choline
		(as % Acetylcholine Rate)		
Human plasm (a) ..	2,700	2 (c)	16	12
Electric organ (b) ..	115	0	—	—
Bovine erythrocyte (a)	$9{\cdot}3 \times 10^4$ (d)	0	1	1

Hydrolysis by Cholinesterases.—The rate of hydrolysis of the new esters by representative cholinesterases is given in Table II. The results show that DMAC is hydrolysed only very slowly by plasma cholinesterase and negligibly by red-cell and electric organ cholinesterases. The other two unsaturated esters were hydrolysed slowly by plasma cholinesterase and just detectably by red-cell cholinesterase. The low rate of hydrolysis of crotonoylcholine by human plasma cholinesterase compared to its optimum substrate, butyrylcholine, shows that the introduction of a double bond into the acyl group greatly retards hydrolysis.

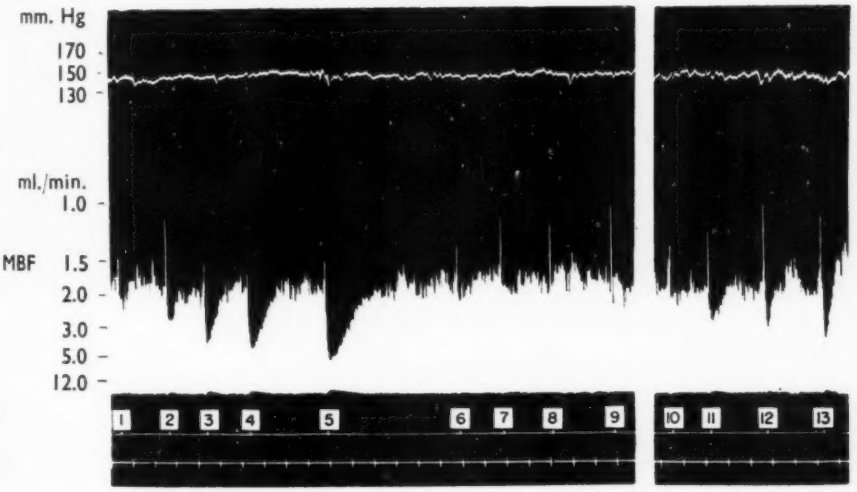


FIG. 9.—Effect of pent-2-enoylcholine and crotonoylcholine, compared with acetylcholine, on blood pressure and muscular blood flow (MBF) in the right hind limb recorded in the femoral artery. Cat, 2·2 kg. Anaesthetic, allobarbitone. Time, min. The following injections were made intra-arterially in 0·1 ml. saline. 1, saline alone; 2 to 5, acetylcholine (2, 0·001 μg.; 3, 0·01 μg.; 4, 0.1 μg.; 5, 1 μg.); 6 to 9, pent-2-enoylcholine (6, 0·1 μg.; 7, 1 μg.; 8, 10 μg.; 9, 100 μg.); 10 to 13, crotonoylcholine (10, 0·1 μg.; 11, 1 μg.; 12, 10 μg.; 13, 100 μg.).

DISCUSSION

DMAC and the two other unsaturated choline esters appear to be the first unsaturated aliphatic choline esters to be investigated pharmacologically, though γ-crotonic betaine esters (Burgen and Hobbiger, 1949), hydroxyalkenyltrimethylammonium compounds (Jacob,

Marszak, Bardisa, Marszak-Fleury, and Epsztein, 1952) and diethylaminoethyl esters of acrylic and senecioic acids (Gilman, Heckert, and McCracken, 1928) have received attention. The new esters are ganglion stimulating and neuromuscular blocking agents; unlike the methyl ester of γ-crotonic betaine they show little or no parasympathomimetic or smooth muscle stimulating action. The new esters are also respiratory stimulants. This action was particularly marked in the case of DMAC and was localized by denervation experiments to the carotid sinus region. Their relative potencies as respiratory and ganglion stimulating agents run parallel; whether this is fortuitous or not is not known, since it is uncertain whether the respiratory stimulation exerted by ganglion stimulating agents is due to a direct action on the chemoreceptors or to a reduction in the carotid blood flow brought about by sympathetic stimulation as suggested by Daly (1954).

The neuromuscular blocking action of the new esters is probably of the depolarizing type, as evinced by the potentiation of the blockade by eserine. Further, Thesleff (personal communication) has found that DMAC and murexine produce a short-lasting membrane depolarization when applied close to a sensitive motor end-plate spot on the isolated tenuissimus muscle of the cat by the ionophoretic micro-application technique (Castillo and Katz, 1957). The depolarization was more prolonged than that produced by acetylcholine. DMAC, like murexine, is only very slowly attacked by cholinesterases, so that it is unlikely that its short-lasting action or the potentiation of its effect by eserine can be ascribed to interactions with cholinesterases. This is in accord with the conclusions reached by Hansson (1958) and Jacob *et al.* (1952) for suxamethonium and other compounds.

Our investigations, thus far, have been concerned only with the mammalian pharmacology of DMAC and throw no light on the rôle of the ester in the hypobranchial gland of *Thais floridana*. The close resemblance between the mammalian pharmacology of murexine and DMAC may, however, extend to their invertebrate pharmacology, and suggests that the rôle of the two esters in their respective species is identical.

One of us (V. P. W.) wishes to express his warm appreciation of the kindness of Professor B. Uvnäs in allowing him to work in the Department of Pharmacology of Karolinska Institutet, and his grateful thanks to the Swedish Medical Research Council, and to AB Vitrum for financial support.

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Brit. J. Pharmacol. (1958), **13**, 315.

ABSENCE OF RENAL ACTION OF PURE INTERMEDIATE LOBE HORMONE

BY

R. B. CROSS

From the Department of Physiology, the University, Edinburgh

(RECEIVED MAY 9, 1958)

The action of melanophore expanding hormone of the intermediate lobe of the pituitary (MSH) on the urinary excretion of water, Na, K and Cl was studied in the conscious dog both during water diuresis and in the non-diuretic state. Doses of up to 2.3 IU MSH did not influence renal function. MSH did not modify the renal actions of vasopressin or oxytocin. The antidiuretic action of a massive dose of MSH was attributed to contamination by vasopressin.

Commercial preparations of oxytocin have been observed to have an action on renal function which is not exhibited by highly purified natural oxytocin. Working with the conscious dog, Brooks and Pickford (1957) reported that, during water diuresis, highly purified oxytocin exerted no effect other than to cause a moderate antidiuresis while the same dose of commercial extract in about 60% of cases interrupted the diuresis and caused a simultaneous increase in the absolute quantities of Na, K and Cl present in the urine. This electrolyte response was similar to, but of greater magnitude than, that caused by an antidiuretic dose of vasopressin. Brooks and Pickford were unable to reproduce this action of commercial oxytocin by contaminating purified natural oxytocin with purified natural vasopressin. This suggested the existence of a third substance, present as a contaminant which either increased electrolyte excretion by itself or modified the effect of one or both of the known posterior lobe hormones.

The melanophore expanding hormone of the intermediate lobe (MSH) is a possible contaminant of all pituitary extracts and, when first extracted, was thought to possess potent antidiuretic activity (Sulzberger, 1933). This property was, however, later attributed to the presence of residual posterior pituitary hormone and purified MSH extracts were declared to be without antidiuretic activity (Fraser, 1937; Landgrebe and Waring, 1941). As there do not appear to have been any extensive investigations into the actions of MSH on electrolyte excretion, this present study was undertaken to determine whether MSH was the active substance which occasionally contaminates commercial oxytocin.

METHODS

A total of 48 observations were made on a young conscious adult bitch of 24 kg. At a preliminary operation the perineum was slit to facilitate catheterization of the bladder. During observations she rested quietly on her side while urine drained into an open measuring cylinder. A T-piece in the catheter close to the vulva enabled the external collecting system to be drained at the end of each collecting period. The dog was trained to contract the bladder when the tail was manipulated. Urinary dead space was therefore minimal.

At 11.45 a.m. the dog was given 250 ml. water by stomach tube and all further food and water withheld until 2.15 p.m. when a water diuresis was provoked by the administration of 300 ml. water by mouth. The bladder was drained subsequently at 10 min. intervals for 2 hr. The test substances were made up to a volume of 1.5 ml. in 0.9% NaCl, and administered intravenously about 45 min. after the water loading. When observations were to be made on the non-diuretic state, the foregoing timetable was adhered to but with the omission of the water loading at 2.15 p.m. Na and K were measured by an EEL flame photometer and Cl was estimated by the method of Prout Winter (Cole, 1919).

The MSH used was from a sample prepared by Dr. J. Porath, using the method of Porath, Roos, Landgrebe, and Mitchell (1955). Subdivisions of this sample were made by first dissolving in distilled water, dividing by volume, and as rapidly as possible freeze-drying and sealing in a vacuum. Each batch of subdivisions was tested for activity on an isolated frog skin and at the end of the series of experiments a sample was submitted to Dr. B. Hobson for accurate assay by the method of Landgrebe and Waring (1944). The vasopressin used was highly purified arginine vasopressin prepared by Dr. V. du Vigneaud. The oxytocin used was the synthetic product Syntocinon

made by Messrs. Sandoz and the commercial extract was the Pitocin of Messrs. Parke, Davis & Co. The doses of vasopressin and oxytocin used were of the same order as those used by Brooks and Pickford (1957). The dose of MSH was estimated as something grossly in excess of any contaminating level in commercial pituitary extracts.

Results

Throughout the experiments the pattern of electrolyte excretion remained fairly uniform. The initial level of both Na and K excretion usually lay between 30 μ equiv./min. and 50 μ equiv./min. with the K level usually the lower. Both levels fell progressively during the diuresis in the manner reported by Ali, Cross, and Pickford (1958). The Cl level usually began about 20 μ equiv./min. above the Na level but fell more rapidly during the early stages of the diuresis so that the subsequent course of the Cl excretion often corresponded fairly closely with that of the Na excretion. Initial levels of Na excretion as low as 15 μ equiv./min. and as high as 100 μ equiv./min. were observed, but these were due to known errors of diet. Results obtained from experiments carried out at these extreme levels of electrolyte excretion did not differ from those obtained when the experiments were repeated at the intermediate levels of electrolyte excretion.

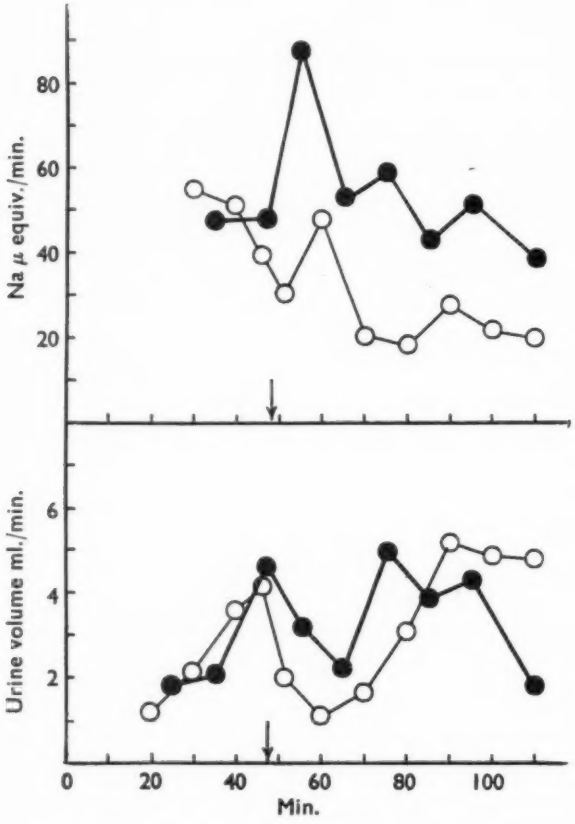

FIG. 1.—Comparison of the effects of synthetic oxytocin and commercial oxytocin upon water and Na excretion during water diuresis in a dog. 300 ml. water was given at 0 time. Intravenous injections were made at 48 min. (arrow). Solid circles, Pitocin 150 mU., and open circles, synthetic oxytocin 150 mU.

Oxytocin Alone.—During the non-diuretic state both synthetic oxytocin and Pitocin caused an increase in electrolyte excretion without any corresponding alteration in urine flow. During a water diuresis a dose of 150 mU. synthetic oxytocin was found to decrease slightly the urine output and to cause a transitory interruption in the downward trend in electrolyte excretion. The same dose of Pitocin caused an inhibition of diuresis with a simultaneous increase in Na and Cl excretion (Fig. 1).

MSH Alone.—MSH was administered alone by single injection in doses of 10 mU., 70 mU., 140 mU., and 2.3 IU both in the diuretic and non-diuretic state and did not alter the expected pattern of water and electrolyte excretion. A priming dose of 70 mU., followed by 100 mU. infused during 15 min., was also without effect during a diuresis. On one occasion a massive dose of 100 IU administered during a diuresis inhibited the diuresis and increased the rate of electrolyte excretion. This response was similar to that expected from a dose of vasopressin of the order of 2 mU.

MSH plus Vasopressin.—A dose of 2 mU. vasopressin administered during a water diuresis was found to cause a transient fall in urine volume with a simultaneous increase in electrolyte excretion. This response was not altered by the addition of either 70 mU. or 2.3 IU MSH to the vasopressin. During the non-diuretic state, vasopressin neither alone nor in combination with MSH altered the pattern of excretion of water and electrolytes.

MSH plus Oxytocin.—MSH in doses of 70 mU. and 2.3 IU administered in combination with 150 mU. synthetic oxytocin did not alter the action of the oxytocin either in the diuretic or non-diuretic state.

MSH plus Vasopressin and Oxytocin.—A mixture of 0.6 mU. vasopressin and 80 mU. synthetic oxytocin was found to inhibit the diuresis and to increase the Na excretion by 15/20 μ equiv./min. for 15 min. The action of this mixture was not modified by the addition to it of either 10 mU. or 2.3 IU MSH.

Discussion

The above findings would appear to dispose of the suspicion that MSH was responsible for the unexpected actions of commercial oxytocin observed by Brooks and Pickford (1957). The doses of MSH used in this study varied from small ones to those grossly in excess of any expected level of contamination in commercial extracts. Indeed, the preparation used by Brooks and Pickford (Pitocin) was reported by Landgrebe and Waring (1950) to be free of MSH, and a more recent assay using a method sensitive to 1 mU. failed to detect any MSH in a dose of Pitocin equivalent to 300 mU. oxytocin (Hobson, personal communication).

There is also no evidence that MSH exerts any direct influence on the excretion of water and electrolytes. The one occasion on which a massive dose of 100 IU MSH inhibited a diuresis and increased the output of electrolytes can probably be attributed to the presence of contaminating vasopressin. This dose was administered during studies into the excretion of MSH, and when compared with the dose levels required for pigment change in amphibia appears to be well beyond physiological limits. Although the extract was stated to contain less than 0.001% vasopressin (Porath, personal communication), such a level of contamination might explain the findings in this case.

It may be that some action of MSH on renal function would have appeared had the observations been continued beyond the 60 to 90 min. periods used in this study, but short as this time is it represents at least three times the circulating life of an injected dose of MSH (Landgrebe and Waring, 1941). Lerner, Shizume and Bunding (1954) administered massive doses of MSH to human subjects daily for 36 days. Unfortunately they did not investigate urinary excretion of electrolytes, but their studies of serum electrolytes did not reveal a significant deviation from normal. Since any gross changes in the urinary excretion of electrolytes if maintained for 36 days might be expected to cause some secondary alteration in serum electrolyte levels, the failure to observe these changes suggests that the prolonged administration of MSH did not alter renal function to any great degree.

I desire to thank Dr. M. Pickford, who made available the samples of MSH and vasopressin from supplies kindly provided by Dr. J. Porath and Dr. V. du Vigneaud. Dr. Pickford also prepared the perineum of the dog. I also wish to thank Dr. B. Hobson, of the Pregnancy Diagnosis Laboratory of the University of Edinburgh, who carried out the assay of MSH. This work was undertaken while holding the Wilkie Research Fellowship of the University of Edinburgh.

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ACTION OF DIETHYLCARBAMAZINE *IN VITRO* ON INFECTIVE LARVAE OF *WUCHERERIA BANCROFTI*

BY

P. JORDAN

From the East African Institute for Medical Research, Mwanza, Tanganyika

(RECEIVED MAY 15, 1958)

Infective larvae of *Wuchereria bancrofti* were obtained from laboratory infected *Culex fatigans* and maintained in serum containing diethylcarbamazine. There was no difference in the survival time of these larvae and larvae maintained in control serum without diethylcarbamazine. In one of the three experiments reported the survival time of the larvae was much longer than in the other two experiments. It is suggested that this may have been due to the presence of filarial antibodies which killed the larvae in two of the sera used. In the serum which did not contain antibodies, the larvae survived very much longer.

Little work appears to have been done on the effect of diethylcarbamazine on infective larvae of *W. bancrofti*. Hawking, Sewell and Thurston (1950) found evidence of a lethal effect *in vivo* against infective larvae of *Litomosoides carinii* when the drug was given to cotton rats before and during the period of exposure to infected mites (*Liponyssus bacoti*). When given after infection, it appeared that developing males may be affected.

McGregor and Gillies (1956) suggested that the follow-up results obtained after diethylcarbamazine therapy in Gambia indicated that the drug had little effect on the ovaries of developing *W. bancrofti*.

In view of the paucity of information regarding the effect of diethylcarbamazine on infective larvae, a series of experiments to assess the effect of this drug on infective larvae of *W. bancrofti* was carried out. If the drug did have an effect on this stage of the life-cycle of the worm it might be of use as a causal prophylactic.

MATERIALS AND METHODS

Preliminary experiments indicated that infective larvae, extracted from *Culex fatigans* dissected in human sera, could be kept alive in capillary tubing for several days. Menon and Ramamurti (1941) had kept larvae alive in whole blood, changed daily, for 8 days, but considered that bacterial infection was responsible for the worms dying.

Laboratory bred *Culex fatigans* were fed on a volunteer showing microfilariae of *W. bancrofti* in his night blood. The mosquitoes which fed were kept at approximately 28° and at a relative humidity of between 80% and 100%. When infective larvae had developed the mosquitoes were dissected and the larvae picked up in capillary tubing.

The use of sterile materials—slides, capillary tubing, and dissecting needles—and the addition of penicillin to the sera in which the mosquitoes were dissected, reduced the risk of bacterial infection and enabled some larvae to remain alive for as long as 16 days. The mosquitoes themselves, however, could not be sterilized.

Lubran (1950) showed that the blood concentration of diethylcarbamazine reached a maximum approximately 4 hr. after an oral dose. A dose of 10 mg./kg. body weight produced maximum blood values of 4 to 8 μg./ml. Two hr. after 15 mg./kg., a blood value of 8.7 μg./ml. was obtained in one case. In order to obtain maximum blood concentrations in the sera to be used against the infective larvae, 500 mg. of diethylcarbamazine citrate, repeated in 3 hr., was given to 3 volunteers, one African and two Europeans. One European complained of lassitude, nausea, and vertigo after the first dose. The total dose of drug/kg. of body weight in the three experiments was 10.6 mg., 16.6 mg., and 16.6 mg. respectively. Prior to the first dose, blood was taken from each person and the sera from this specimen was used as a control. Cystalline sodium benzylpenicillin was added in a dose of approximately 400 units/ml. of serum in an endeavour to limit bacterial growth.

The mosquitoes were immobilized by stunning and dissected in the sera, control and drug containing sera being used alternately. Any infective larvae seen were taken up into capillary tubing. If there were many larvae in the mosquito, more than one sometimes inadvertently entered a tube. Care was taken to avoid any infective larvae which appeared to be damaged in any way even though their movements might suggest that the damage was unimportant.

The column of serum containing the infective larva was shaken to the middle of the tube. The ends were sealed with plasticine, which also served for the purpose of fixing the tube to a numbered slide. Under the microscope, the infective larva could be seen in the tube and its movements noted. The tubes were kept at room temperature (22° to 24°).

The activity of the larva was noted two or three times in 24 hr. When it failed to move on three consecutive occasions, it was considered to be dead. It was frequently found that after being apparently dead at one and sometimes two observations, the larva would be obviously alive when observed the next time.

In spite of care being taken to avoid damaged larvae, some may have been injured and died in a few hours. It was impossible to say whether these early deaths were due to injury, drug, or natural causes. In an endeavour to exclude those which were injured, larvae apparently dead when first examined, and which failed to move at the next two examinations, were considered to have been damaged in some way. These have been excluded from the results. Others, living only 2 hr., have accordingly not been excluded, but the possibility of their deaths being due to damage cannot be ruled out. The time at which the larva was taken into the tube and the time at which the larva was last seen alive were used for calculating the length of life of each larva to the nearest hour. The mean length of life of the larvae in the different sera was then calculated.

Results

The results are given in Table I, from which it will be observed that there was a considerable range in the length of life of infective larvae in all sera.

In no experiment was there any significant effect of the drug on the mean life of the larvae. In Expt. 1, in which the concentration of diethylcarbamazine was lower than in the other experiments (see above), there was an apparent shortening of the life of the larvae, while in Expt. 2 the drug appeared to increase the length of life of the larvae.

It appears from these results that diethylcarbamazine has no lethal effect *in vitro* on infective larvae. If a higher dose of drug was given there might be some effect, but the doses given were near the upper limit of tolerance and it would be impracticable to give a higher dose regularly for prophylactic purposes.

These *in vitro* results cannot be taken to indicate that the drug would be of no use prophylactically. In very few cases has this drug shown any *in vitro* effect on the different stage of filarial worms (Hawking *et al.*, 1950), and it may well be that *in vivo* the drug acts indirectly by rendering the larvae more susceptible to phagocytosis.

It will be seen from Table I that the mean length of life of the infective larvae in the different experiments varied considerably.

Since it has been established that the drug had no effect on the larvae, it is possible to combine the results for the control sera and the sera containing drug in each experiment (Table I).

It is seen that the mean length of life of infective larvae in Expt. 3 was nearly double that of the larvae in Expts. 1 and 2, and this difference is statistically significant.

Since the same technique was used in the three experiments it is interesting to speculate on the possible reason for this result. There seem to be two possible explanations. First, the serum for Expt. 1 was obtained from a European with many years' exposure to filarial infection. This person was known to have a positive filarial complement fixation test. The serum used in Expt. 2 was from an African who, although showing no clinical signs of filariasis, had lived all his life in an endemic area. It is therefore very probable that the sera used in the first two experiments contained antibodies.

Serum used in Expt. 3 was from a European who had only been in East Africa for a short time and who had not been exposed to filarial infection. There would, therefore, be no antibodies in the sera, and the infective larvae might thus be expected to live longer in this serum than in the sera of the other two experiments.

The second reason for the longer life of larvae in Expt. 3 concerns the age of the larvae. In

TABLE I

THE MEAN LENGTH OF LIFE OF INFECTIVE LARVAE IN CONTROL SERA, SERA CONTAINING DRUG, AND THE TWO SERA COMBINED

The mean length of life is measured to the nearest hour.

Expt.	Control				Sera with Drug				Control and Sera with Drug Combined			
	No. of Larvae	Mean Length of Life (hr.)	Range	S.E.	No. of Larvae	Mean Length of Life (hr.)	Range	S.E.	No. of Larvae	Mean Length of Life (hr.)	Range	S.E.
1	79	34	2–155	±3	94	27	2– 81	±4	173	31	2–155	±2
2	51	23	8–69	±2	63	37	8–106	±3	114	31	8–106	±2
3	68	57	4–320	±8	60	56	5–238	±6	128	57	4–320	±5

this experiment they were removed from the mosquitoes 18 days after the infected blood meal; in Expts. 1 and 2, this time interval was 14 and 16 days respectively. If the age of the larvae was responsible one would expect a graduated increase of the survival time in the three experiments.

Further work is required to determine the influence of antibodies in this type of study, but some support for the antibody explanation given above comes from an earlier experiment in which the survival times of infective larvae maintained in sera from a newly-arrived European and from an African with elephantiasis were compared. The survival time of the larvae in the European serum was significantly greater than that of the larvae in the serum of the African.

When further work on these lines has been done, it may be possible to devise a new serological test for filariasis which might be more specific than those at present in use.

Grateful acknowledgments are made to the Administrator, E.A. High Commission, for permission to publish this work, and to Mr. Francisco Baloe and Mr. Albinus Mahunde for help in dissecting many hundreds of mosquitoes.

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THE EFFECT OF SUBSTANCE P ON THE PERISTALTIC REFLEX OF THE ISOLATED GUINEA-PIG ILEUM

BY

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The introduction of substance P into the lumen of the isolated guinea-pig ileum caused an increase in the number and amplitude of the peristaltic waves. In preparations in which the peristaltic reflex was abolished, by fatigue, by external or internal application of 5-hydroxytryptamine, or by lowering the temperature of the bath, the introduction of substance P into the lumen of the intestine restored peristalsis. This effect of substance P was absent in preparations in which the mucous membrane was removed. Hexamethonium abolished the effect of substance P on peristalsis. It is concluded that substance P acts on the afferent nervous elements of the peristaltic reflex arc, possibly on the sensory receptors.

Substance P has been found in relatively high amounts in the intestine and brain of various animals, such as the monkey, horse, ox, pig, sheep, dog, cat, rabbit and man (v. Euler and Gaddum, 1931; Douglas, Feldberg, Paton and Schachter, 1951; Ehrenpreis and Pernow, 1952; Pernow, 1953). A factor having, qualitatively and quantitatively, the same properties as substance P has been shown to occur in the intestine of the cod and the dogfish and in cod brain (v. Euler and Östlund, 1956). The retina of the cow has also been found to contain high amounts of substance P (Duner, v. Euler and Pernow, 1954).

The exact physiological role of substance P is still unknown. A central stimulating effect of substance P has been shown by v. Euler and Pernow (1954), and evidence has been presented that substance P stimulates afferent fibres (Lembeck, 1957). In evaluating the action of substance P on the jejunum of the rabbit, Blair and Clark (1956) concluded that the stimulant action of substance P on intestinal motility is mediated through a very labile mechanism which could well have a trophic influence on the amplitude of spontaneous contractions.

It is known that the peristalsis elicited by increased intraluminal pressure is augmented in the presence of substance P (Gernandt, 1942). In the present experiments the effect of substance P on the peristaltic reflex was studied, introducing the substance into the lumen of the guinea-pig ileum.

MATERIALS AND METHODS

For recording the peristaltic activity a modification of the method of Trendelenburg (1917) was used, which has been described in detail in a previous paper (Beleslin and Varagić, 1958). By this method it was possible to introduce the drug into the lumen of the intestine and to wash it out. The peristaltic activity was recorded by means of Stephenson's float recorder which records volume changes (Stephenson, 1948).

The peristaltic reflex was tested by raising the intraluminal pressure (varying from 30 to 50 mm. in different experiments) for 90 sec. at constant intervals.

Several experiments were done with preparations from which the mucosa had been removed. For this purpose a loop of intestine was turned inside out by slipping it over a glass rod. The mucosa was gently scraped off using a piece of filter paper, and then the loop of intestine was returned to its normal position. As a control for these experiments another piece of ileum was subjected to the same procedure but without scraping off the mucosa.

The intraluminal injections were made in a volume of 0.1 to 0.2 ml. The intestine was kept in Tyrode solution which was oxygenated with a mixture containing 97% O_2 and 3% CO_2. The volume of the bath was 20 ml.

The drugs used were 5-hydroxytryptamine creatinine sulphate, hexamethonium bromide and nicotine hydrogen tartrate. Two samples of substance P were used, one containing 2.6 units/mg. and the other 3 units/mg.

RESULTS

Substance P and Normal Peristalsis.—It has been shown that substance P may potentiate the peri-

stalsis produced by increased intraluminal pressure. Gernandt (1942) found that, in the presence of substance P, peristaltic activity was produced by an otherwise subliminal pressure and that at higher pressure peristaltic activity was increased. Similar results were obtained in the present experiments. Substance P was found to potentiate peristalsis when introduced into the lumen of the intestine. This potentiation was evident by an increase in the number or amplitude of the peristaltic waves. In a preparation in which fatigue was allowed to develop, substance P produced vigorous peristalsis. A typical experiment is shown in Fig. 1. The normal peristalsis is shown in *a*. In *b* the effects of fatigue and of substance P are shown. The intraluminal pressure was raised and was kept high for some time. It can be seen that fatigue developed after 5 min. and peristaltic activity stopped. At the first arrow 0.2 ml. of Tyrode solution was introduced into the lumen of the gut and it produced two peristaltic waves. At P, 10 units of substance P was introduced into the lumen of the intestine. Substance P caused the appearance of vigorous peristalsis which lasted for 15 min. with no sign of fatigue. Between *b* and *c* the intestine and the bath were washed out, after which the emptying phase of the peristaltic reflex was almost abolished, as shown in *c*.

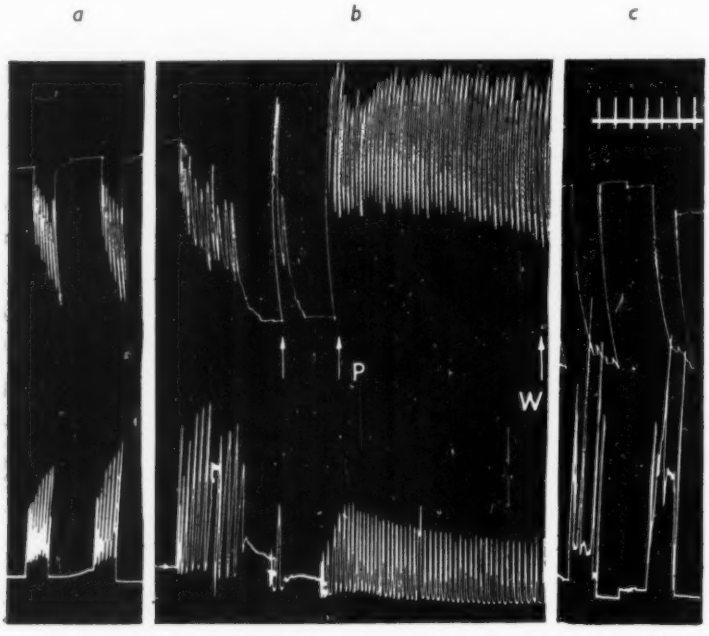

FIG. 1.—Effect of substance P on fatigued guinea-pig ileum. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. *a*, two normal peristaltic responses to a raised intraluminal pressure. *b*, at first arrow, 0.2 ml. Tyrode solution injected intraluminally (i.l.); at P, 10 units substance P i.l.; at W, bath and intestine washed out. *c*, 110 min. after washing. Time, 1 min.

Effect of Lowering the Bath Temperature.—It has been shown that cooling the guinea-pig ileum to temperatures between 19° and 26° abolished the emptying phase of the peristaltic reflex (Kosterlitz and Robinson, 1957; Beleslin and Varagić, 1958). The addition of 5-hydroxytryptamine (5-HT) in low concentrations to the fluid outside the intestine restored slight peristaltic activity after this activity had been abolished by cooling. The introduction of 5-HT into the lumen of the cooled gut regularly restored or increased peristaltic activity (Beleslin and Varagić, 1958). We have now found that the introduction of substance P into the lumen of the cooled intestine also caused the reappearance of peristalsis. A typical experiment is shown in Fig. 2. The normal peristaltic reflex at 37° is shown in *a*. Between *a* and *b* the temperature of the bath was lowered to 24°. At this temperature the peristaltic reflex was abolished. At P in *b*, 10 units of substance P was introduced into the lumen of the gut. The immediate effect of the substance P was a slight rise in the tone of both the circular and longitudinal muscles. After a latent period of 13 min., vigorous peristalsis occurred. This activity lasted for 10 min. with no sign of fatigue. The bath and the intestine were then washed out at W. Twenty min. later, raising the intraluminal pressure caused no peristalsis.

Substance P and 5-HT.—5-HT added to the bath at 37° in concentrations from 10^{-6} to 10^{-5} g./ml. will depress or abolish the peristaltic reflex (Kosterlitz and Robinson, 1957; Ginzel, 1957). It is now shown that substance P may antagonize this effect of 5-HT. At H in Fig 3*a*, 1 mg. of 5-HT was put into the bath, making the final concentration 5×10^{-5} g./ml. This drug was allowed to act for 1 min. and then the intraluminal pressure was raised. This caused only one peristaltic wave followed by a complete inhibition of the peristaltic reflex. At P, 10 units of substance P was introduced into the lumen of the gut. After a latent period of 3 min. this dose of substance P caused vigorous peristalsis. Between Figs. 3*a* and *b*, the bath and the intestine were washed out. Twenty-five min. after washing out 5-HT and substance P, the peristaltic reflex was still abolished, but it had recovered 140 min. after washing (Fig. 3*b*).

After the saturation of the tryptamine receptors by the introduction of 5-HT into the lumen of the gut, a similar observation was made. Fig. 4 shows the effect of 5-HT and substance P on a fatigued guinea-pig ileum. The intraluminal injection of 400 ng. of 5-HT caused the reappearance of peristalsis which lasted for 120 sec., as shown at the first H. The second intraluminal injection of the same dose of 5-HT produced only three peristaltic waves. The third intraluminal injection of the same dose of 5-HT had no effect, as shown at the third H. The introduction of 20 units of substance P into the lumen of the gut now caused vigorous peristalsis lasting 5 min., as shown at P.

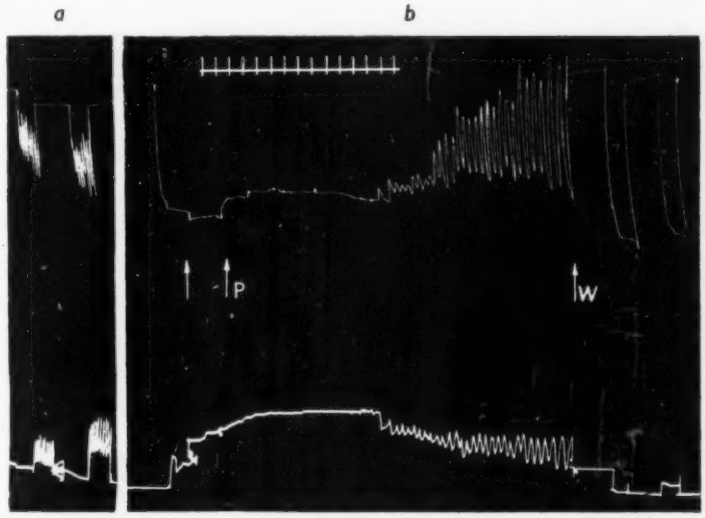

FIG. 2.—Effect of substance P on cooled intestine. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. *a*, two normal peristaltic responses at 37° to raised intraluminal pressure. Between *a* and *b* the temperature of the bath was lowered to 24°. *b*, at first arrow, 0·2 ml. Tyrode solution i.l.; at P, 10 units substance P i.l.; at W, bath and intestine washed out. Time, 1 min.

Effect of Hexamethonium.—The contractions of the longitudinal muscle of the guinea-pig ileum caused by substance P are not blocked by ganglion-blocking agents. It was therefore concluded that the action of substance P is probably due to a direct action on the smooth muscle fibres (Pernow, 1953). In the present experiments, it is shown that the effect of substance P on peristalsis can be abolished by hexamethonium. The effect of 10 units of substance P, introduced intraluminally, on the fatigued intestine is shown in Fig. 5*a*. At C_e 0.4 mg. (2×10^{-7} g./ml.) hexamethonium was added to the bath and produced a complete abolition of the effect of substance P.

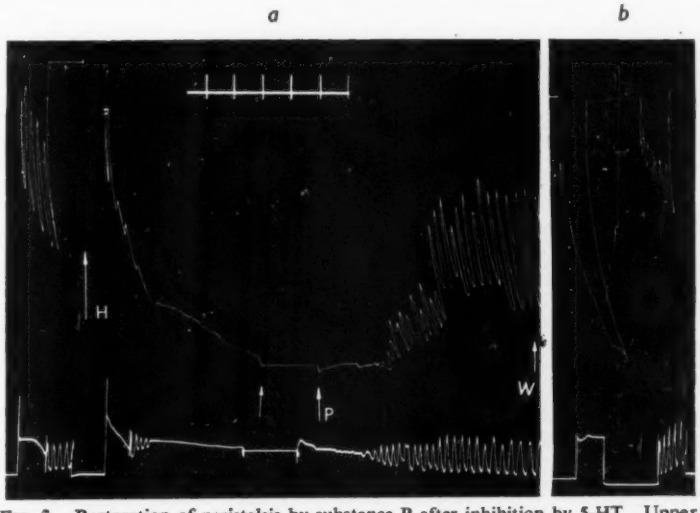

FIG. 3.—Restoration of peristalsis by substance P after inhibition by 5-HT. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. *a*, at H, 1 mg. 5-HT added to bath; at arrow, 0·2 ml. Tyrode solution i.l.; at P, 10 units substance P i.l.; at W, bath and intestine washed out. *b*, responses obtained 25 and 140 min. after washing out. Time, 1 min.

Effect of Removing the Mucous Membrane.—In a separate series of 9 experiments substance P was tested on pieces of intestine from which the mucous membrane had been removed. The peristaltic reflex was abolished in all 9 preparations. This is in accordance with the finding of Bülbring, Lin and Schofield (1958). The introduction of 10 units of substance P into the lumen of an intestine from which the mucous membrane had been removed did not cause peristalsis to return. This was the case in all 9 experiments. In 7, nicotine caused a contraction of the gut, showing that a ganglionic response could be obtained although substance P failed to produce peristalsis. The effect of substance P in a fatigued preparation is shown in Fig. 6*a*. The effect of substance P on a preparation from which the mucous membrane had been removed is shown in Fig. 6*b*. Although substance P failed to produce peristalsis, nicotine (at G) caused a contraction of the longitudinal muscle.

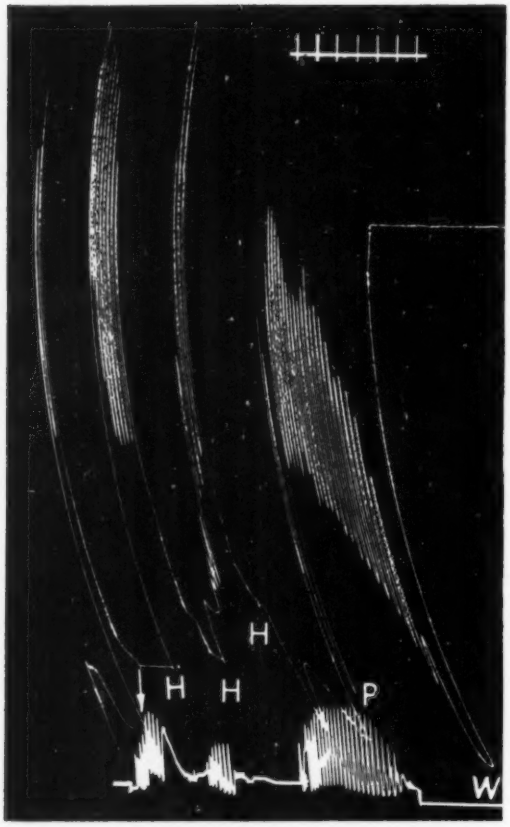

FIG. 4.—Effect of 5-HT and substance P on peristalsis. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. At arrow, 0·2 ml. Tyrode solution i.l.; at H, 400 ng. 5-HT i.l.; at P, 20 units substance P i.l. Time, 1 min.

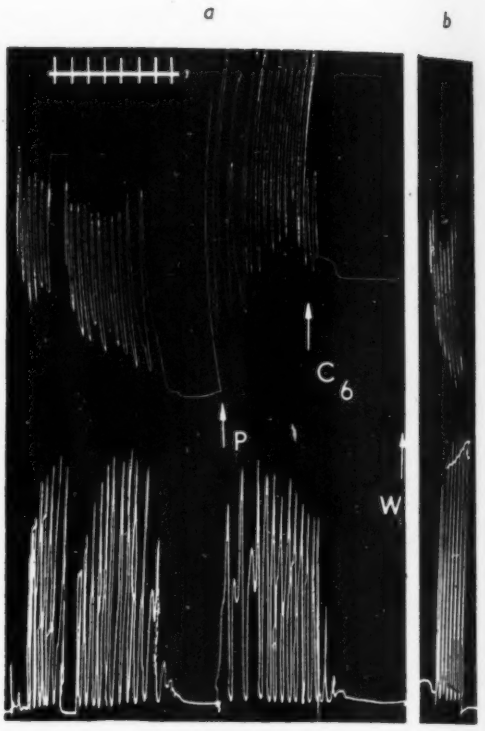

FIG. 5.—Effect of hexamethonium (C_6) on peristalsis produced by substance P. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. *a*, at P, 10 units substance P i.l.; at C_6, 0·4 mg. C_6 added to bath; at W, bath and intestine washed out. *b*, 45 min. after washing. Time, 1 min.

DISCUSSION

Substance P potentiates peristalsis which occurs in response to increased intraluminal pressure. Local and intravenous application of small amounts of substance P may increase the tone and rhythmic spontaneous activity of the small intestine *in situ* (Gernandt, 1942). The results of the present experiments are in accordance with these findings. In addition, it was found that the introduction of substance P into the lumen of the fatigued intestine gave rise to vigorous peristalsis. In some experiments the period of increased peristaltic activity was followed by a partial or almost complete abolition of the emptying phase of the peristaltic reflex.

The present experiments show that substance P may produce peristalsis when the tryptamine receptors have been saturated by a large dose of 5-HT added to the bath or introduced into the lumen of the gut. The receptors for substance P may therefore differ from those for tryptamine.

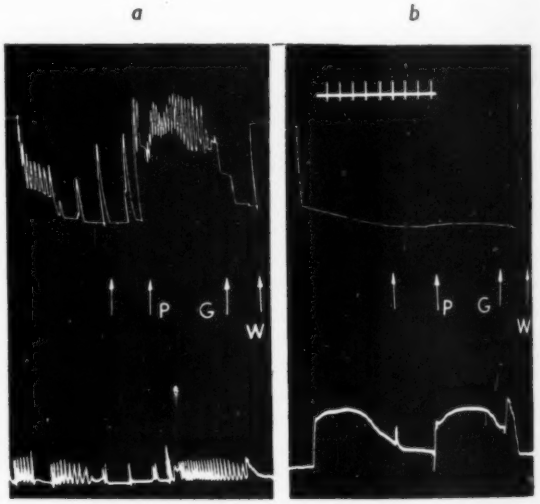

FIG. 6.—Effect of removing mucous membrane on response to substance P. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. *a*, control preparation. *b*, preparation from which mucous membrane had been removed. At first arrow, 0·2 ml. Tyrode solution i.l.; at P, 10 units substance P i.l.; at G, 0·75 mg. nicotine into bath; at W, bath and intestine washed out. Time, 1 min.

The experiments with hexamethonium show that substance P acts at two different sites. A direct effect of substance P on the smooth muscle fibres has been postulated by Pernow (1953). The present experiments show that substance P may also act on the preganglionic nervous elements of the reflex arc. It was possible in some experiments to differentiate between these two actions of substance P. Thus the addition of hexamethonium to the bath stopped the contractions of the circular muscle and left the contractions of the longitudinal muscle intact. Substance P has been found to stimulate the afferent nervous fibres in the rabbit ear (Lembeck, 1957). The present experiments suggest that substance P stimulates the afferent fibres of the peristaltic reflex arc. The experiments with the intestine from which the mucous membrane was removed seem to support this view. On the other hand these experiments also indicate that substance P may act on the sensory receptors in the mucosa. Whether the action of substance P is part of a physiological process required for peristalsis is not proved by these experiments. Medaković and Radmanović (personal communication) have found that during peristalsis a substance (or substances) is liberated into the lumen of the guinea-pig ileum which contracts the guinea-pig ileum and fundus of the rat stomach. The effect of this substance cannot be abolished by atropine or antazoline (Antistin) and only occasionally can it be abolished by 2-bromo-lysergic acid diethylamide.

In some experiments the effect of substance P was only seen after a latent period. This period was found to be considerably longer when the substance P was introduced into the lumen of the cooled intestine. It is possible therefore that substance P may act through another active substance which it liberates, and that the process of liberation is affected by changes in temperature.

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THE CHROMATOGRAPHIC BEHAVIOUR OF WASP VENOM KININ, KALLIDIN AND BRADYKININ

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Wasp venom kinin which has hitherto appeared to be homogeneous can be resolved by ion-exchange chromatography into a single major and two minor components. These are indistinguishable by their action on smooth muscle and by their rapid inactivation by chymotrypsin. All three components of wasp kinin are chromatographically different from kallidin or bradykinin. The close similarity of the latter compounds is confirmed by their identical behaviour on an ion-exchange resin.

Wasp venom kinin, kallidin, and bradykinin are substances with many chemical and pharmacological properties in common. Wasp kinin occurs with histamine and 5-hydroxytryptamine in the venom of the common European wasp, *Vespa vulgaris* (Jaques and Schachter, 1954; Schachter and Thain, 1954). Kallidin and bradykin (termed plasma kinins by some authors) are derived by the actions of kallikrein (Werle, 1955), or of snake venoms and trypsin (Rocha e Silva, 1955) on an α_2 globulin of blood plasma. All three substances appear to be polypeptides. They produce a characteristic slow contraction of isolated smooth muscle, are vasodilator agents, increase capillary permeability and cause pain when applied to the exposed blister base of human skin (Holdstock, Mathias, and Schachter, 1957). The present experiments are concerned with the chromatographic behaviour of wasp kinin and of kallidin and bradykinin.

METHODS

Preparation of Wasp Kinin, Kallidin, and Bradykinin.—Wasp venom kinin was prepared by extracting dried wasp venom glands with acetic acid; the kinin was precipitated by the addition of ether, washed repeatedly with small amounts of ethanol, taken up in water and freeze-dried. In several instances the material was, in addition, dialysed against 10 to 20 volumes of water in cellophane sacs with continuous shaking (dialysate changed hourly for 3 hr.). Bradykinin and kallidin were prepared by the action of crystalline trypsin and human salivary kallikrein respectively on heated ox serum globulin. Details of these procedures are described by Holdstock *et al.* (1957). The bradykinin preparation contained 1 unit (Rocha e Silva, Beraldo and Rosenfeld, 1949) in 140 μg. of dry material; 120 μg. kallidin was equivalent in activity on the guinea-pig ileum to 1 unit of bradykinin.

Chromatography. — Ascending paper chromatograms were run at room temperature on Whatman No. 1 paper and the eluates tested on the isolated rat uterus and guinea-pig ileum as previously described (Holdstock *et al.*, 1957). The solvents used were: (*a*) water (56%)/ammonium sulphate (20%)/ "cellosolve" (24%) as described by Martin and Porter (1951); "cellosolve" in ethylene glycol mono-ethyl ether; (*b*) ethanol/0.2N-HCl; and (*c*) *n*-butanol/acetic acid/water (Partridge, 1948). The chlorine-starch-iodide spray reaction was performed as described by Rydon and Smith (1952) except that the time of exposure of the paper to chlorine was shortened from 10 min. to 1 min. The azocarmine test was carried out according to Turba and Enenkel (1950).

By the methods outlined by Hirs, Moore, and Stein (1953), we found that wasp kinin was completely absorbed on the resin, Amberlite (XE-64), from solution in a phosphate buffer (Gomori, 1955) at *p*H 6. At higher *p*H values, however, kinin partitioned between the buffer and resin and a *p*H of 7.7 was considered satisfactory for ion-exchange chromatography. The resin was prepared as described by Hirs *et al.* (1953), and equilibrated with 0.1M phosphate buffer (*p*H 7.7) containing thymol, and packed in a glass cylinder as a column 0.9 × 25 cm. Wasp kinin (300 to 500 μg.) was dissolved in 0.5 ml. of the buffer (0.05 ml. was kept beside the column and 0.05 ml. frozen, as controls), slowly added to the column and washed on with a small amount of buffer. Elution was continued with the same buffer at a flow rate of approximately 3 ml./hr. The eluate was collected in fractions of 0.94 ml. which were frozen

at regular intervals. The tubes were later thawed and tested for pharmacological activity. Bradykinin (9.0 mg.) and kallidin (8.0 mg.) were chromatographed in the same way.

Isolated Smooth Muscle Preparations.—Rat uterus or guinea-pig ileum was suspended in an 18 ml. bath at 26 to 28° and 34 to 36° respectively. Atropine and mepyramine were present in the bath throughout with the isolated guinea-pig ileum, and atropine and lysergic acid diethylamide (LSD) with the rat uterus; the final concentration of each of these drugs in the bath was 10^{-4} g./l.

Drugs and Other Reagents.—Atropine was used as sulphate, mepyramine as maleate, and (+)-lysergic acid diethylamide as tartrate. Trypsin and chymotrypsin were crystalline, salt-free preparations (Armour).

RESULTS

Paper Chromatography

The R_F of wasp kinin has been either zero or very low in all solvents hitherto employed for its chromatography on paper (Holdstock *et al.*, 1957). In the present experiments, however, we have succeeded in obtaining a high R_F value in two solvents. These were ammonium sulphate-cellosolve and ethanol-hydrochloric acid (see Methods). Most experiments were carried out with the former. Pharmacological testing of eluates on the isolated rat uterus or guinea-pig ileum revealed activity only in a single well-defined band with an R_F approximately 0.45 in both solvent systems. The region on the paper which corresponded to the eluted pharmacological activity was devoid of ultra-violet absorbing or fluorescent material. It did, however, react very faintly with ninhydrin, strongly with chlorine-starch-iodide reagent and particularly strongly with azocarmine. In fact, this was the only area stained by azocarmine with chromatographed wasp kinin prepared as described above and dialysed for a limited time. There were, however, at least two pharmacologically inactive bands of higher R_F values showing positive reactions to

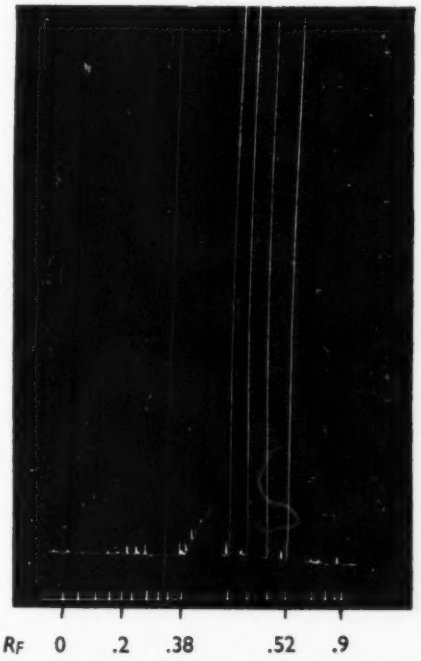

FIG. 1.—Contractions of isolated guinea-pig ileum in the presence of atropine and mepyramine (each 10^{-4} g./l.) to eluates of two-dimensional paper chromatogram of wasp kinin. The R_F value are those in the second solvent $(NH_4)_2SO_4$-cellosolve.

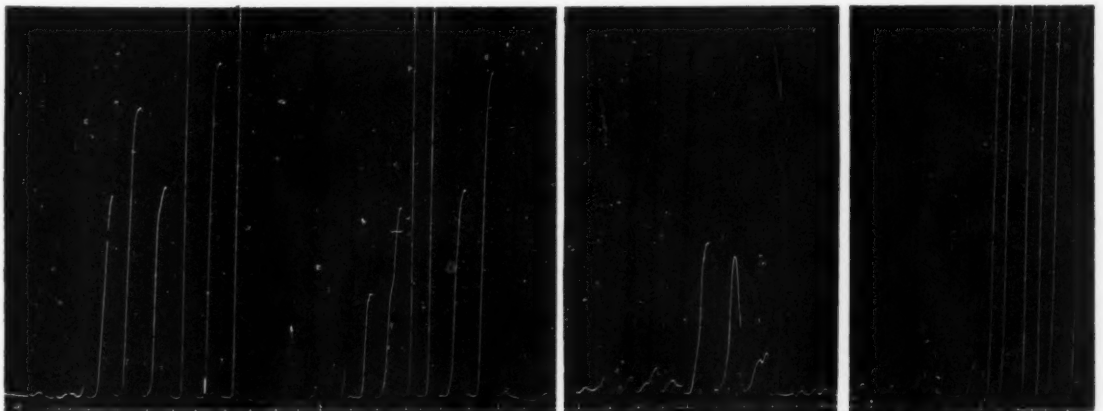

FIG. 2.—Contractions of isolated rat uterus in the presence of atropine and LSD (10^{-4} g./l.) to fractions from the chromatography of wasp kinin on Amberlite (XE-64). Minor peaks 11 to 21 and 29 to 41; major peak 109 onwards has been cut short at 121. Trace activity (in this experiment only) in fractions 73 to 77.

ninhydrin and to chlorine-starch-iodide. Two-dimensional chromatograms were also run using butanol-acetic acid as the first solvent and ammonium sulphate-cellosolve as the second. Elution of the paper yielded only a single pharmacologically active area in the position expected from unidimensional chromatography (Fig. 1) with a recovery of 30 to 40%. This area reacted strongly with azocarmine. The positive reaction of the pharmacologically active material with the chlorine-starch-iodide and azocarmine reagents supports the view that it is a polypeptide.

Bradykinin and kallidin could not be separated by paper chromatography in the ammonium sulphate-cellosolve solvent. These substances moved somewhat faster than wasp kinin with an R_F value of approximately 0.5. Recoveries were approximately 25%.

Ion-exchange Chromatography

Five experiments in which wasp kinin of varying purity was applied to the ion-exchange resin Amberlite (XE-64) yielded identical results. In all experiments there were two minor (but definite) peaks of activity emerging from the column rapidly and close to one another, and a delayed major peak (Fig. 2). The major peak which appeared in fractions 100 to 110 and persisted beyond fraction 140 contained approximately 85

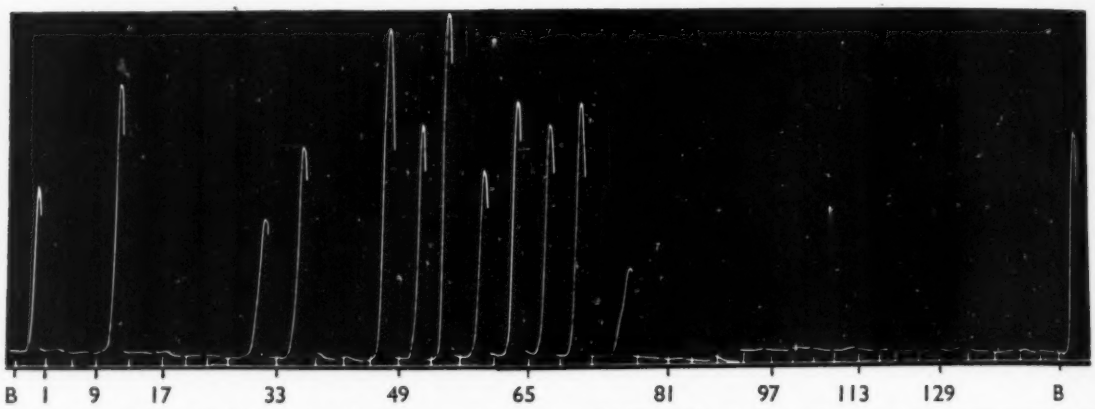

FIG. 3.—Contractions of isolated rat uterus in the presence of atropine and LSD (10^{-4} g./l.) to fractions from the chromatography of bradykinin on Amberlite (XE-54). B, bradykinin standard.

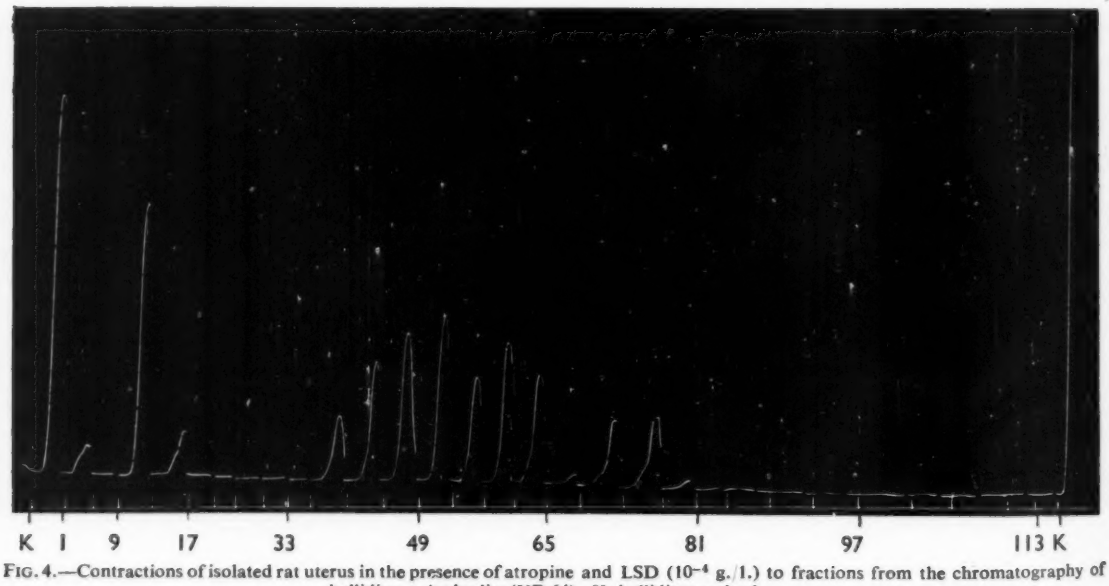

FIG. 4.—Contractions of isolated rat uterus in the presence of atropine and LSD (10^{-4} g./l.) to fractions from the chromatography of kallidin on Amberlite (XE-64). K, kallidin standard.

to 90% of the activity recovered in the eluate. The total recovery of activity was approximately 30% at room temperature and 50% or more when chromatography was carried out at 4°. All three peaks possessed the same relative activity on both the guinea-pig ileum and rat uterus preparations. Also, the three activities were completely destroyed by incubation for 20 min. at 37° with crystalline chymotrypsin and much reduced by trypsin (25 μg./ml.). The fractions corresponding to each of the three peaks were bulked, dialysed for a limited time against water to reduce the concentration of buffer salts, and each of the three peaks rechromatographed individually. We failed, however, to recover activity in this way and it would appear that wasp kinin(s) becomes less stable in a purified state. Attempts to remove the buffer salts with the resin "Biodeminrolit" (Permutit) proved unsuccessful because kinin was also completely adsorbed on the resin.

Kallidin and bradykinin were also chromatographed at room temperature in the same way. The behaviour of these two substances on the resin was almost identical. The main bradykinin peak emerged in fractions 44 to 72 (Fig. 3) and that of kallidin in 38 to 73 (Fig. 4). In the case of bradykinin there was also a minor activity in fractions 29 to 33. Both kallidin and bradykinin show a very narrow band of activity in tube 9 which corresponds to the hold-up volume of the column. This activity is probably due to traces of the major active material washed out by impurities which pass freely through the column. The recoveries of bradykinin and kallidin were approximately 25% and 15% respectively.

Part of the expenses of this work were defrayed by a grant from the Central Research Fund of the University of London which is gratefully acknowledged.

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Brit. J. Pharmacol. (1958), **13**, 330.

A METHOD OF ASSAYING THE ANTI-TETANUS POTENCY OF DRUGS ON EXPERIMENTAL LOCAL TETANUS IN THE RABBIT

BY

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(RECEIVED JANUARY 30, 1958)

Local tetanus was induced in rabbits by the intramuscular injection of tetanus toxin. The relative potency of drugs which abolished the muscle spasm was determined using quantitative electromyography and the accuracy of the method tested with mephenesin and thiopentone. It is suggested that the method is suitable for screening new drugs for clinical use.

There is no general agreement on the best method of controlling the convulsions of clinical tetanus, which still carries a high mortality rate.

One of the reasons for this lack of agreement is the absence of information as to which drugs possess desirable anticonvulsant properties in tetanus. These properties are an ability to suppress the convulsions without interfering with respiration and without causing loss of consciousness.

Clinical tetanus is now usually treated with general anaesthetics and hypnotics or with neuromuscular-blocking agents, but control can only be achieved at the price of respiratory depression or unconsciousness with the former and respiratory paralysis with the latter. In both cases the treatment promotes complications which may be fatal.

Mephenesin has been widely used in clinical tetanus because it has the required properties to some degree, but it is impotent in the most severe cases and has other disadvantages. However, the existence of mephenesin suggests that a search for more effective drugs would be worth while. The first requirement for such a search is a method for quantitative comparison of drug activity and this forms the subject of the following report.

METHOD

Rabbits in which local tetanus had been induced in one hind limb by injection of tetanus toxin into the gastrocnemius muscle were suspended in a canvas sling so that their hind limbs just touched the bench.

The electrical activity of the developed tetanus was recorded with intramuscular electrodes placed 10 to 15 mm. apart with an indifferent electrode attached to the skin of the back. The recorded potentials were amplified by a condenser-coupled pre-amplifier and a main amplifier and displayed on an oscillograph (Wright, Morgan, and Payling Wright, 1952). These voltages were then summed by an integrating circuit to an arbitrary level at which a pulse was emitted to a "Dekatron" counter. Every 100th pulse so obtained was led off through a relay circuit and signal marker to a smoked drum. Thus a certain degree of muscular activity was converted into a quantitative unit and the number of units was directly proportional to the degree of electrical activity.

It was found, however, that spontaneous local tetanus was too variable to give a consistent activity from which small deviations could easily be measured, and so an electrically operated hammer was arranged to strike the frame supporting the animal in its sling at intervals of 1 min. With this

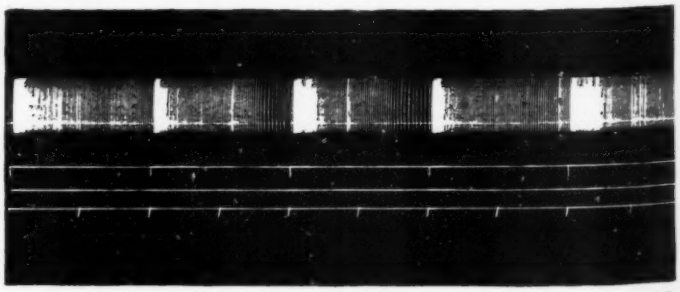

FIG. 1.—Record of activity from the affected muscle of a rabbit with experimentally induced local tetanus prior to drug injection. Upper tracing, muscle activity; middle tracing, applied stimulus; lower tracing, injection signal line; time, 30 sec. Note the burst of activity coincident with each applied stimulus and then the gradual decline in activity towards the end of the minute.

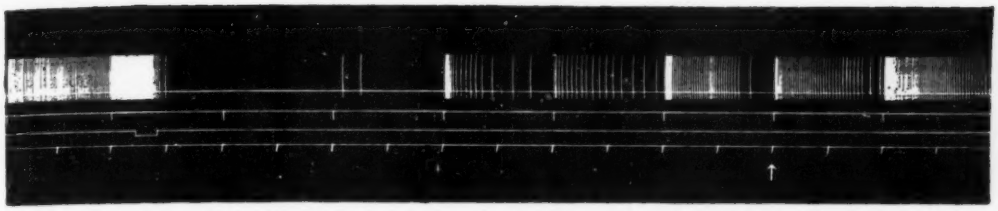

FIG. 2.—Record of the effect of 15 mg./kg. mephenesin (at signal) on the activity of the affected muscle of a rabbit with experimentally induced local tetanus. Upper tracing, muscle activity; middle tracing, applied stimulus; lower tracing, injection signal line; time, 30 sec. In this instance the end of the drug effect, based on the arbitrary criteria chosen, is indicated by the arrow. The duration of abolition of tetanus is 5½ min.

standard afferent stimulus it was possible to achieve a more steady state (see Fig. 1).

In order to minimize the effect of the physical disturbance of making the injection in the ear, the base of the ear was infiltrated with 2.0 ml. of 2% procaine before each experiment. This amount of procaine did not affect the tetanus.

Before the administration of any drug, tetanus activity was recorded until a base-line of 5 min. constant activity was achieved. The experimental drug was then injected intravenously and a quantitative measure of the potency obtained as follows. The beginning of a drug effect was taken from the time all activity ceased. The drug effect was considered to have finished when a minute was reached in which activity, starting coincidentally with the stimulus, was maintained so that at least 10 units of activity were recorded in the second half of that minute. Thus in Fig. 2 the end of the drug effect is indicated by the arrow and the response time is 5½ min.

If further doses were to be given the animal was left undisturbed for 10 min. before reapplying the stimulus preparatory to the next injection.

Mephenesin and thiopentone, both drugs known to be active against clinical tetanus, were given in a Latin square arrangement of four doses. The rows in each Latin square represented different days of the experiments, the columns different times of day when the doses were given. Each square was then carried out on one rabbit.

TABLE I

DOSE/RESPONSE RELATIONSHIP FOR ABOLITION OF TETANUS ACTIVITY BY MEPHENESIN

The values above the broken line are the results of a Latin square of four doses performed on one rabbit on four consecutive days. Those below the broken line represent a similar experiment performed on another rabbit.

Dose (mg. kg.) ..	10	15	20	30
Response (min.) ..	4·5	5·0	6·75	7·5
	2·0	5·0	6·5	10·5
	3·5	4·75	5·5	15·0
	3·25	4·25	5·75	7·0
	3·0	5·25	6·0	11·5
	3·5	5·5	6·5	9·5
	3·0	3·75	5·5	10·0
	3·0	3·75	5·0	9·5
Mean ..	3·22	4·66	5·94	10·06

RESULTS

Dose/Response Relationship

Mephenesin.—The results of two complete Latin squares for mephenesin are shown in Table I. The mean responses, when plotted against dose, were found to give a linear regression that extends through zero (Fig. 3).

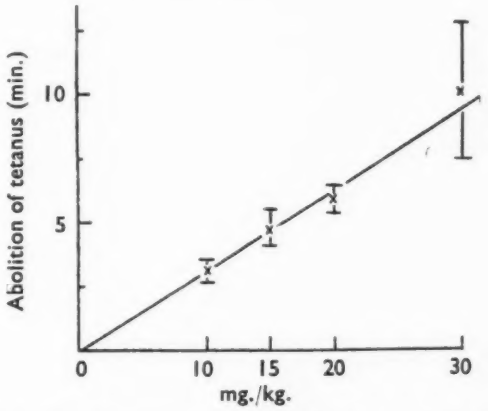

FIG. 3.—Dose response relationship for the anti-tetanus activity of mephenesin. Each point is the mean of eight observations and the standard deviations are also shown.

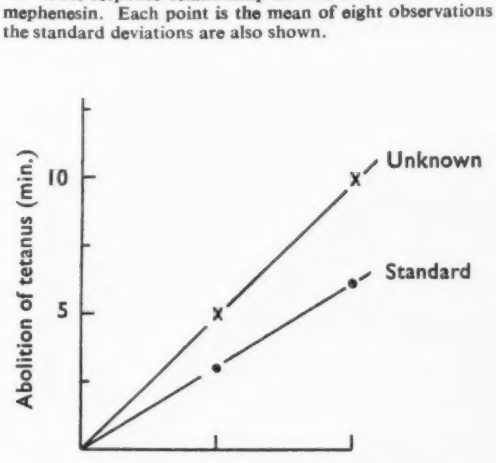

FIG. 4.—Slope ratio assay of the anti-tetanus activity of mephenesin. With the unknown, the doses used were 15 and 30 mg./kg., while with the standard, 10 and 20 mg./kg.

If two of the doses (10 and 20 mg./kg.) are treated as standard and two (15 and 30 mg./kg.) as unknown, then their two regression lines are not parallel but intersect at a point for which the dose is zero (Fig. 4). The relative potency of the two solutions is then the ratio of the slopes of the two regression lines (Wood, 1951). The regression coefficients $b_u . b_s$ can be calculated and the relative potency of the two solutions $= b_u / b_s = R = 1.625$. The theoretical value $= 1.5$, the "unknown" doses being 50% greater than the "standard" doses. The 5% limits of error, calculated according to Burn (1950), were 1.31 and 1.94.

Thiopentone.—The results of two similar Latin squares for thiopentone, shown in Table II, were treated as for mephenesin (Figs. 5 and 6). The relative potency of the two solutions $= b_u / b_s = R = 1.32$, with limits of error ($S = 0.05$) of 1.10 and 1.54. The theoretical value of the ratio is, in this case, 1.33.

TABLE II

DOSE/RESPONSE RELATIONSHIP FOR THE ABOLITION OF TETANUS ACTIVITY BY THIOPENTONE

See Table I for an explanation of the arrangement of results.

Dose (mg./kg.) ..	6	8	12	16
Response (min.) ..	3·5	5·0	5·25	9·0
	2·5	6·5	10·5	13·5
	3·5	6·5	12·5	8·5
	3·5	4·5	7·5	10·5
	3·0	6·25	8·0	14·0
	4·0	4·25	8·5	18·0
	9·0	7·0	14·0	14·5
	8·0	7·0	11·0	18·0
Mean ..	4·625	5·875	9·660	13·25

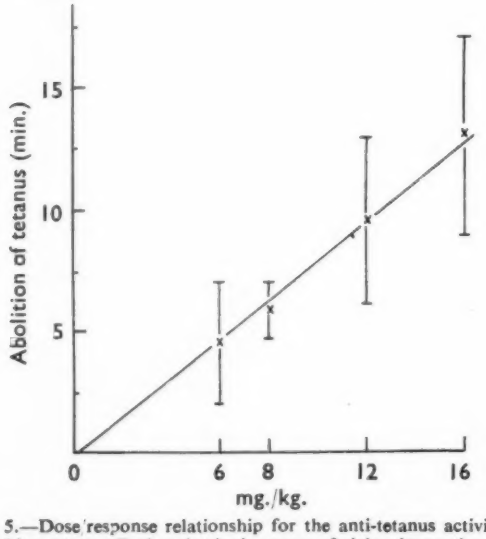

FIG. 5.—Dose/response relationship for the anti-tetanus activity of thiopentone. Each point is the mean of eight observations and the standard deviations are also shown.

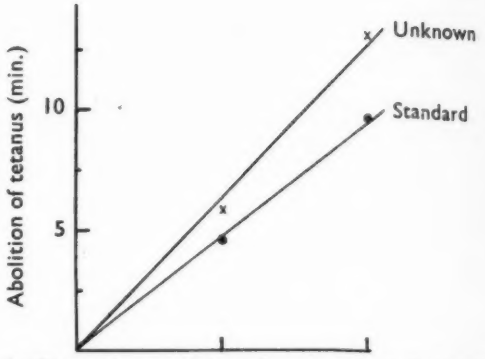

FIG. 6.—Slope ratio assay of the anti-tetanus activity of thiopentone. With the unknown, the doses used were 8 and 16 mg./kg., while with the standard, 6 and 12 mg./kg.

Analysis of Variance

The significance of a number of the possible sources of error in this assay can be established statistically. They are: (1) A systematic variation in the response of the animals with the order in which the doses are given. (2) A daily variation in the overall response of each rabbit depending on the stage of tetanus reached. (3) A variation in the sensitivity of different animals to the same drug.

The four doses were given to each rabbit in a Latin square arrangement covering four days so that all doses were given each day, and during the period of the experiment the four possible dose orders were covered. Thus an analysis of variance performed on the combined results for the two squares can be made to determine the significance of the three sources of error. From the results of the analysis shown in Tables III and IV it will be seen that: (1) The order in which the doses are given is not important. (2) There is no significant daily fluctuation in the response of the experimental animals. (3) There is a significant difference in the overall response of the different animals to the same drug in the case of thiopentone.

TABLE III

SLOPE/RATIO ASSAY FOR MEPHENESIN

Analysis of variance of the results in Table I.

Nature of Variation	Sum of Squares	d.f.	Mean Square	F.
Between doses	208·36	3		
Regression	207·11	2	103·44	57·340
Non-convergence at zero dose	1·25	1	1·25	
Between experiments	12 812	7		
Between animals	0·195	1	0·195	
Between days within animals	12 617	6	2 103	1·166
Dose order (between columns)	7·047	3	2·349	1·234
Error	32 48	18	1·804	
Total ..	260·7	31		

TABLE IV

SLOPE RATIO ASSAY FOR THIOPENTONE
Analysis of variance of the results in Table II.

Nature of Variation	Sum of Squares	d.f.	Mean Square	F.
Between doses	365·09	3		
Regression	364·077	2	182 04	32 741
Non-convergence at zero dose	1·013	1	1·013	
Between experiments	103·885	7		
Between animals	53·854	1	53·854	9·515
Between days within animals	50·031	6	8·3385	1·499
Dose order (between columns)	11·454	3	3·818	0·687
Error	100·071	18	5·560	
Total ..	580·50	31		

DISCUSSION

The results reported show that this assay is sufficiently sensitive to differentiate between small changes in dose of mephenesin and thiopentone and therefore may reasonably be used to screen drugs for their anti-tetanus activity.

However, with thiopentone the assay is obviously at its limit of discrimination because a reversal of results with closely spaced doses occurred on one occasion (Table II). Differences in the rate of return of tetanus after a drug are liable to cause this occasional reversal where the lower dose appears to have a greater effect than the higher. This is due mainly to the relative insensitivity introduced by the arbitrarily selected end-point. Steps are being taken to remove this anomaly, which has only occurred with thiopentone, although it is rare enough not seriously to affect the assays in which it arises.

It is possible that the greater scatter of responses with thiopentone than with mephenesin is due to differences in their site of action.

The established difference in the response of different animals to the same drug means that whenever possible the comparison of different drugs should be performed on the same rabbits.

We should like to thank the Wellcome Research Laboratories for the preparation of tetanus toxin, British Drug Houses for the supply of mephenesin, and May & Baker Ltd. for the thiopentone. We are indebted to Dr. H. O. Schild for statistical advice, Dr. O. C. J. Lippold for advice on the apparatus, and D. Sayers for technical assistance. The salary of one of us (R. A. W.) and part of the cost of the apparatus was generously donated by May & Baker Ltd.

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THE ACTIVITY OF A VARIETY OF CHEMICAL COMPOUNDS AGAINST EXPERIMENTAL TETANUS

BY

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(RECEIVED MAY 21, 1958)

A range of chemical compounds, mostly with central nervous system depressant activity, have been tested against experimental tetanus in the rabbit. A number of the more potent tested, including mephenesin, betanaphthoxyethanol, barbiturates and phenothiazine derivatives, have been accurately assayed by a method of quantitative electromyography. Phenothiazine derivatives were found to be the most potent anticonvulsants and of these acetylpromazine had the greatest activity. The difficulties of direct comparison of the potency of substances from different chemical groups is discussed.

Tetanus is a disease in which generalized convulsions, if uncontrolled, are an important, although by no means the sole, cause of death. The convulsions may be controlled by continuous neuromuscular block with artificial respiration or by sedation. Most sedative drugs, however, when used in adequate anticonvulsant dosage, cause either loss of consciousness or respiratory depression, or both. The ideal drug would arrest convulsions without these effects.

The survey of various central nervous depressants reported here was undertaken to find which series of compounds most closely approaches the ideal, and to obtain some quantitative comparison of the relative potency of the compounds tested. In this way it was hoped to discover drugs likely to be of use in clinical practice and to compare them with drugs in current use.

Choice of Drugs

The drugs chosen for accurate assay, together with the reasons for the choice, are given below.

Mephenesin.—The spinal cord and brain-stem depressant activity of mephenesin makes it theoretically an ideal drug for use in tetanus, and many examples of its use have been reported. It is, however, active for only a very short time and has other disadvantages.

Betanaphthoxyethanol.—This compound has central actions similar to mephenesin and may also have some additional anaesthetic effect which might enhance its anti-tetanus activity.

Barbiturates.—The barbiturates have had extensive use in the treatment of tetanus.

Phenothiazines.—Chlorpromazine is known to have marked anti-tetanus activity in both animals and man.

Other substances were chosen because they were known to have paralysing effects (for example benzimidazole derivatives), were related chemically to the above-mentioned drugs or had been tried or suggested for trial in clinical tetanus in the past.

METHOD

The method employed for producing and recording experimental local tetanus in the rabbit was that of Laurence and Webster (1958). Local tetanus was induced in rabbits by an injection of tetanus toxin into the gastrocnemius muscle of one hind limb. The electrical activity of this muscle was recorded with intramuscular electrodes and the potentials amplified and monitored on an oscillograph. These voltages were summed by an integrating circuit to a level at which an impulse was fed to a Dekatron counter. Every 100th pulse was then led off through a relay circuit to either a signal marker on a smoked drum, or an electromagnetic counter. The electrical activity of the tetanized musle was thus converted into arbitrary units and the anti-tetanus activity of a drug determined by a chosen criterion. The criterion originally used had a number of shortcomings that have been discussed (Laurence and Webster, 1958). In the present work, the % reduction of tetanus activity for a 20 or 30 min. period immediately after administration of the drug was used as the index of potency. A control count of the electrical activity from the tetanus muscle was made each minute for a 5 or 10 min. period, depending on the variability of the tetanus in the individual animal. An example of the derivation of the value for the % reduction of activity is shown in Fig. 1. All the drugs were given intravenously.

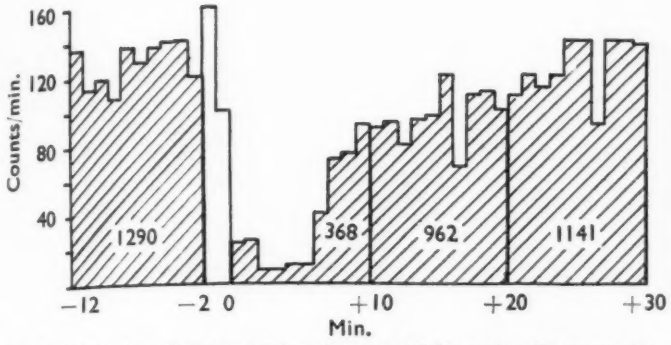


FIG. 1.—Derivation of the % reduction of tetanus activity produced by promazine. The ordinate is a count of the electrical activity /min. from the affected muscle of a rabbit with local tetanus. Total counts/10 min. periods are also shown. The 2-min. injection period is not included in the calculation. Between −2 and 0 min., 0·25 mg./kg. of promazine was injected intravenously. The % reduction of tetanus activity was calculated as follows. % Reduction $= 100-\left(\frac{368+962+1,141}{3\times 1,290}\times 100\right)=36\%$.

RESULTS

Comparison of Some Chemical Groups Known to Have Anti-tetanus Activity

The drugs used were mephenesin, thiopentone, chlorpromazine, and betanaphthoxyethanol.

The assay was designed to extend over four days, during which time each animal had two doses of all four compounds. The doses used (Fig. 2) were those found in preliminary experiments to give results that straddled the 50% reduction line. The reduction of activity was calculated for the 20 min. period following the injection. The dose order was randomized so that as far as possible any variation in the level of tetanus was nullified.

Not all the animals in which experimental tetanus was induced could be used in the assay, as in some cases a workable level of tetanus activity was never achieved.

The results of this assay are shown in Fig. 2. The plotted values for mephenesin and betanaphthoxyethanol are the mean results from nine animals. With chlorpromazine and thiopentone, however, the results from only six animals have been used. This is because the doses of these two compounds originally chosen, and used on the first three animals, were too high and gave results that were not comparable with those for the other drugs. Subsequently lower doses of chlorpromazine and thiopentone were used.

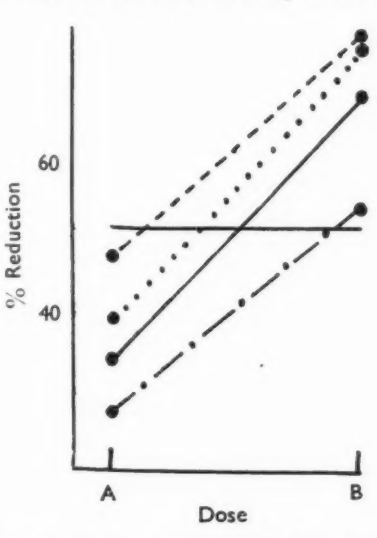


FIG. 2.—Mean results for the comparison of the anti-tetanus activity of .—.—. mephenesin (15 and 30 mg./kg.); ——— betanaphthoxyethanol (5 and 10 mg./kg.); — — — — thiopentone (5 and 10 mg./kg.); . . . . . chlorpromazine (0·08 and 0·16 mg./kg.). The % reduction of tetanus activity was calculated for the 20-min. period following the injection. A refers to the lower and B to the higher dose given.

TABLE I

A COMPARISON OF THE ANTI-TETANUS ACTIVITY OF SOME CENTRAL NERVOUS DEPRESSANTS

The dose producing a 50% reduction of tetanus activity over the 20-min. period following injection was calculated graphically (Fig. 2).

Compound	No. of Animals	50% Reduction Dose mg./kg.	Activity Ratio
Mephenesin	9	28·5	1
Betanaphthoxyethanol ..	9	7·5	3 8
Thiopentone	6	5·6	5 0
Chlorpromazine ..	6	0·11	266

The relative potencies were calculated by measuring the dose of each drug which produced a 50% reduction of tetanus activity. This dose, together with the activity of each compound, compared with mephenesin, is shown in Table I. From this it will be seen that chlorpromazine is over 200 times and thiopentone 5 times as active as mephenesin.

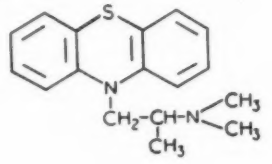
By "*t*" test calculations, all the lines were found to have a significant slope and not to deviate significantly from parallel. Since chlorpromazine was so much more active than the other compounds it was decided to investigate some other phenothiazines.

Comparison of the Anti-tetanus Activity of Some Phenothiazine Derivatives

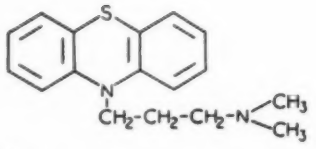
The four most active derivatives of thirteen tested were chosen for accurate assay. They were: chlorpromazine, acetylpromazine, promazine, and

promethazine. Their formulae are shown in Fig. 3. The assay was arranged as before with the exception that the recording period following drug injection was 30 instead of 20 min., as phenothiazine compounds have the highly desirable property of long duration of action.

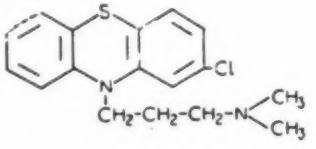
Promethazine



Promazine



Chlorpromazine



Acetylpromazine

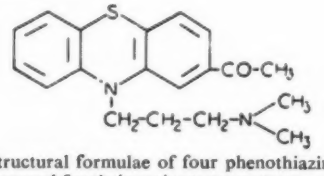


FIG. 3.—The structural formulae of four phenothiazine compounds assayed for their anti-tetanus potency.

The results of this assay for seven animals are shown in Table II and it will be seen that acetylpromazine has ten times the anti-tetanus activity of chlorpromazine. Again, "*t*" test calculations show that for each drug a significant regression line was obtained but that in no case did the lines deviate significantly from parallel.

TABLE II

A COMPARISON OF THE ANTI-TETANUS ACTIVITY OF SOME PHENOTHIAZINES

The dose producing a 50% reduction of tetanus activity over the 30-min. period following injection was calculated graphically. The graphical presentation of the results of this assay is similar to that shown in Fig. 2.

Compound	No. of Animals	50% Reduction Dose mg./kg.	Activity Ratio
Acetylpromazine ..	7	0·019	10·5
Chlorpromazine ..	7	0·20	1·0
Promazine	7	0·44	0·46
Promethazine	7	0·60	0·33

Other Compounds

In addition to those assayed above, the following compounds were tested qualitatively for anti-tetanus activity. These were not assayed accurately as they were obviously ineffective either in comparison with other members of their chemical group as with some phenothiazines; or the group as tested was impotent, for example benzimidazole derivatives. It was not thought worth while to devote to them the time that an accurate assay would have demanded.

The *phenothiazine derivatives* were: diethazine, 10-(2-2-diethylaminoethyl)phenothiazine; ethopropazine, 10-(2-diethylaminopropyl)phenothiazine; pecazine (Pacatal), 10-(1-methylpiperid-3-ylmethyl)-phenothiazine; prochlorperazine (Stemetil), 2-chloro-10-{3-(4-methylpiperazin-1-yl)propyl}-phenothiazine; trimeprazine (6549 RP), 10-(3-dimethylamino-2-methylpropyl)phenothiazine; methotrimeprazine (7044 RP), 10-(3-dimethylamino-2-methylpropyl)-2-methoxyphenothiazine; thiopropazate (SC 7105, Dartal), 2-chloro-10[3-{4-(2-acetoxyethyl)piperazin-1-yl}propyl]phenothiazine; SC 8723, 2-chloro-10-[3-{4-(3-oxobutyl)-piperazin-1-yl}propyl]phenothiazine; SC 8950, 2-chloro-10-{3-(4-methylcarbamoylpiperidino)-propyl} phenothiazine.

Two *benzimidazole derivatives* have been reported to have marked central depressant effects (Domino, Unna, and Kerwin, 1952). They are 2-amino-6-methylbenzthiazole (SKF 1045A) and 2-amino-6-chlorobenzthiazole (I.C.I. 5502). Both of these compounds are less active than mephenesin. Other compounds also tested were: pentobarbitone, hyoscine, benzhexol, benactyzine, caramiphen, diphenhydramine, procaine, morphine, and amphetamine.

DISCUSSION

In evaluating the activity of any drug both the duration and intensity of its effect have to be considered. Whilst giving a comparison which is considered to be sufficiently accurate for screening for possible clinical use, the criterion used in this work is, in effect, a combination of these two factors. It is, however, important to distinguish between these two factors when comparing drugs of different chemical constitution.

Consider two hypothetical substances A and B both exhibiting anti-tetanus activity. They produce, at a certain dosage, a 50% reduction of tetanus activity over a 20 min. period following their injection. Drug A produces total abolition for 10 min. followed by normal tetanus activity for the remaining time, whilst drug B produces a

50% reduction in activity for each of the 20 min. observed. Thus whilst the net result is the same, the means by which it is derived are different and strict comparison of their activities is impractical. To judge the importance of this difference in action in these experiments the 20 min. recording period was divided into 5 min. intervals and the % of tetanus activity, compared with the untreated tetanus, calculated in each instance. The time course of action of the drugs was then compared.

Histograms of the results of these calculations are shown in Fig. 4. From these it will be seen that the type of action of the drugs tested does in fact vary. Chlorpromazine *at the doses used* has a weak prolonged effect (like substance B) whilst the action of mephenesin, although of short duration, is intense, producing sometimes total abolition of tetanus. These graphs also demonstrate the delay in the onset of activity of chlorpromazine.

With the doses used in these experiments, it appears that strict comparison of different chemical compounds by the method employed is not completely practical or fully valid. Mephenesin is apparently only active, particularly at the lower dose level, for about 10 min. and it might be suggested that in order to appreciate the full effect of mephenesin one should compare the drugs over this period only. If, however, the dose of chlorpromazine is increased to produce an effect, in this same time interval, comparable with that of mephenesin, then the action of chlorpromazine is not only evident during this time but extends for

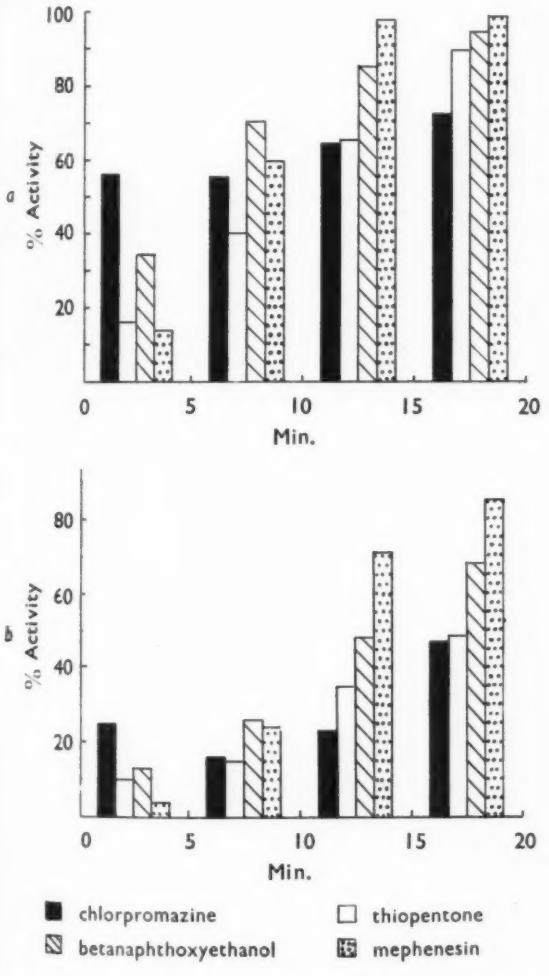


FIG. 4.—Mean time course for the reduction in tetanus activity produced by chlorpromazine (6 rabbits), thiopentone (6), betanaphthoxyethanol (9), and mephenesin (9). The % activity for each 5-min. period following the injection was calculated by comparison with the activity recorded in the control period. In *a*, 0·08 mg./kg. chlorpromazine, 5 mg./kg. thiopentone, 5 mg./kg. betanaphthoxyethanol, and 15 mg./kg. mephenesin were used. In *b*, twice the dose of each compound was given.

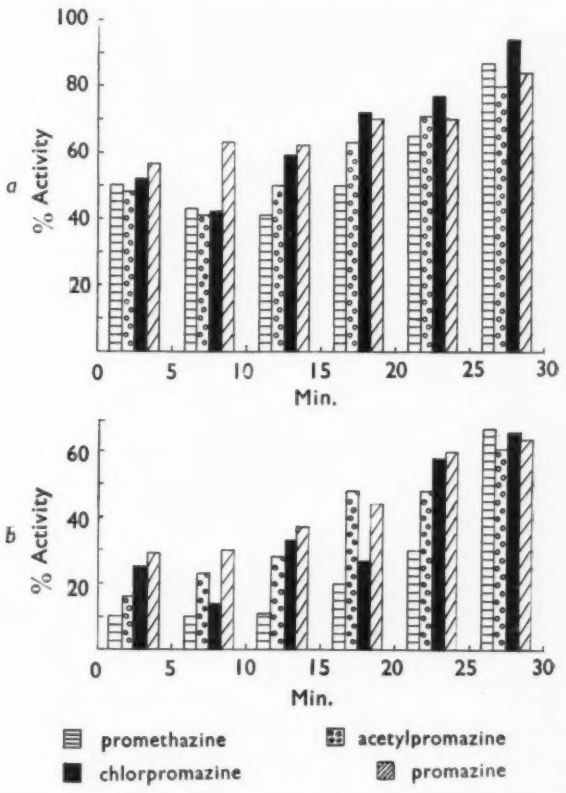


FIG. 5.—Mean time course of the reduction in tetanus activity, in 7 rabbits, produced by promethazine, acetylpromazine, chlorpromazine, and promazine. The % activity for each 5 min. period following the injection was calculated in comparison with the activity recorded in the period immediately before. In *a* 0·5 mg./kg. promethazine, 0·0125 mg./kg. acetylpromazine, 0·125 mg./kg. chlorpromazine, and 0·25 mg./kg. promazine were used. In *b*, twice the dose of each compound was given.

a further 10 min. or more. In fact we would then be failing to record the full effect of chlorpromazine. Bearing these facts in mind the criteria used are a compromise, yet are adequate for the purpose of seeking drugs for clinical use provided the type of action is noted in addition to the simple potency figure.

In Fig. 5 the analysis of the results for the comparative assay of the phenothiazine derivatives is shown. The types of action of all four compounds are sufficiently alike to make strict comparison possible. The delay in onset of action is noticeable.

Phenothiazine compounds are clearly the most effective drugs available at present against tetanus. Chlorpromazine has been used extensively in the treatment of clinical tetanus (Laurence, Berman, Scragg, and Adams, 1958). It has some manifest disadvantages including occasional inability to control severe convulsions.

Arrangements have been made for the clinical trial of acetylpromazine.

The authors wish to thank May & Baker Ltd. for a grant for the salary of R. A. W. and towards the cost of apparatus, as well as for the chlorpromazine, promethazine, and other compounds. The tetanus toxin was kindly supplied by the Wellcome Research Laboratories, Beckenham. Benger Laboratories Ltd., Imperial Chemical Pharmaceuticals Ltd., John Wyeth Bros., G. D. Searle & Co., and Smith Kline & French Laboratories also gave compounds.

We wish to thank Miss S. Schadendorf and Mr. D. Sayers for technical assistance.

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PHARMACOLOGICAL PROPERTIES OF PEMPIDINE (1:2:2:6:6-PENTAMETHYLPIPERIDINE), A NEW GANGLION-BLOCKING COMPOUND

BY

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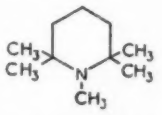
(RECEIVED JUNE 27, 1958)

Pempidine (1:2:2:6:6-pentamethylpiperidine) is a long-acting ganglion-blocking compound which is effective by mouth. By intravenous injection it has a similar potency to hexamethonium on the preganglionically stimulated nictitating membrane of the cat. The compound blocks the effects of intravenous nicotine and of peripheral vagal stimulation on the blood pressure; it also causes dilatation of the pupil after removal of the sympathetic innervation. On the guinea-pig ileum, the predominant effect of the compound is to inhibit nicotine contractions. Pempidine is well absorbed from the gastro-intestinal tract as judged by (*a*) the low ratio (6.9) of oral to intravenous toxicities, (*b*) the rapid development of mydriasis in mice after oral administration of small doses, and (*c*) the rapid onset of hypotension when the compound is injected directly into the duodenum of anaesthetized cats. Other actions include neuromuscular paralysis of curare-like type when large doses of the compound are injected intravenously and central effects such as tremors which occur with near toxic doses. In cats with a low blood pressure, large intravenous doses have a slight pressor action.

Until recently ganglion-blocking compounds of high potency and specificity were thought to be confined to polymethylene bis-quaternary ammonium series, such as hexamethonium (Paton and Zaimis, 1951) and pentolinium (Mason and Wien, 1955). The discovery by Stone, Torchiana, Navarro, and Beyer (1956) that mecamylamine was a potent ganglion-blocking compound revealed that this property was not dependent on the presence of a quaternary nitrogen atom in the molecule. Furthermore, mecamylamine, a secondary amine, was found to be well absorbed from the gastro-intestinal tract and to have a prolonged action.

We have found that several secondary and tertiary amines have potent ganglion-blocking properties and this paper describes the properties of one of them, pempidine (1:2:2:6:6-pentamethylpiperidine).



This compound was examined as a result of an investigation into the structural requirements for orally effective ganglion-blocking compounds with a comparatively long duration of action (Lee, Wragg, Corne, Edge, and Reading, 1958), and it has been described independently by Spinks and Young (1958).

METHODS

Cats were anaesthetized with ether or thiopentone, which was followed by chloralose (80 mg./kg.).

Supramaximal rectangular pulses of 0.5 msec. duration were used to stimulate nerves. In addition to continuous stimulation at a frequency of 10/sec. of the cervical sympathetic nerve trunk, intermittent stimulation at frequencies of 20 or 50/sec. was also applied for 5 sec./min. The peripheral end of the cut right vagus nerve was stimulated at frequencies of 50/sec. The peripheral stump of the cut sciatic nerve of the cat was stimulated at frequencies of 10/min. and 50/sec.

The superior cervical ganglion was perfused by the method of Kibjakow (1933) as used by Emmelin and MacIntosh (1956) using double-dextrose Locke solution containing physostigmine salicylate (10^{-5}). The preganglionic nerve was stimulated at a frequency of 10/sec. for periods of 3 min. and the samples were assayed against acetylcholine chloride on the blood pressure of the eviscerated cat previously treated with mepyramine and physostigmine.

The responses of the pupil of the cat were determined after removal of the ipsilateral superior cervical ganglion (Mason and Wien, 1955).

The isolated guinea-pig ileum was set up in oxygenated Tyrode solution at 37°. Agonists were allowed to act for 30 sec. in a 3 min. cycle and the bath was washed out twice in each cycle. In other

experiments the method of Trendelenburg (1917) was employed.

Langendorff preparations of the rabbit heart were perfused with oxygenated double-dextrose Locke solution at 37°. Drugs were dissolved in the perfusate before injection into the arterial cannula.

The isolated rectus abdominis muscle of the frog was set up in a bath of oxygenated frog Ringer solution at room temperature.

Mydriatic responses were studied in groups of five albino mice (Edge, 1953). The compounds were administered orally.

The effect on nicotine-induced convulsions was studied in groups of 10 albino mice and graphical estimates of the ED50 obtained. Nicotine was injected intravenously 30 min. after an intraperitoneal injection of a ganglion-blocking agent.

The method of Haley and McCormick (1957) was used to study the effect of compounds injected intracerebroventricularly in mice, the drugs being dissolved in 0.9% w/v NaCl solution and injected in volumes of 0.01 to 0.05 ml.

Acute toxicity experiments were performed in groups of 10 albino mice and rats, and the parameters estimated graphically by the method of de Beer (1945).

Pempidine (1:2:2:6:6-pentamethylpiperidine) was used as the hydrochloride (or, in a few experiments, as the hydrogen tartrate). Mecamylamine was used as the hydrochloride, hexamethonium as the bromide, and pentolinium as the hydrogen tartrate. The doses of these four compounds and of adrenaline hydrochloride refer to the cation. The doses of the following compounds refer to their salts unless otherwise stated in the text: decamethonium iodide, tubocurarine and acetylcholine chloride, nicotine hydrogen tartrate, histamine acid phosphate, pilocarpine nitrate, atropine sulphate, mepyramine maleate, physostigmine salicylate, neostigmine methylsulphate, and phenoxybenzamine [benzyl-2-chloroethyl (1-methyl-2-phenoxyethyl) amine hydrochloride].

RESULTS

Action on the Superior Cervical Ganglion of the Cat

An intravenous injection of pempidine or of mecamylamine caused relaxation of the preganglionically stimulated nictitating membrane. This relaxation was slower in development than with hexamethonium (Fig. 1), and, having reached a maximum, recovery was usually very prolonged so that often there was only a very slight recovery over a period of 30 min. or more. In an attempt to follow the course of recovery after an injection, we applied intermittent stimulation to the nerve, but in general it was found that complete recovery of the response did not occur and that a period of rest was required before the response returned. In this respect the experiment illustrated in Fig. 1 is not typical since there was practically complete recovery of the response with no intervening rest period. In this experiment the time required for 50% recovery was, after pempidine, about eight times that for hexamethonium.

The duration of action of pempidine and mecamylamine on the superior cervical ganglion was also relatively prolonged when they were injected into the cannulated lingual artery during occlusion of the external carotid artery.

Having once given an effective intravenous dose of one of these long-acting compounds, a further dose of any ganglion-blocking compound given within a period of 2 to 4 hr. was markedly potentiated. It was therefore difficult to obtain valid graded responses. To determine the potency we first obtained graded responses to hexamethonium and then gave two injections of the long-acting compound separated by a period of at least 2 hr.

It is with these limitations that we assign, on the basis of 13 experiments, a potency to pempidine of 1.35 times that of hexamethonium. In a series of five similar experiments mecamylamine had a potency of 1.22.

Pempidine had no stimulant action on the relaxed nictitating membrane in doses up to 8 mg./kg.

Bennett, Tyler, and Zaimis (1957) found that, after a dose of mecamylamine sufficient to cause a partial block of transmission to the nictitating membrane, tetanic stimulation of the preganglionic

FIG. 1.—Cat, 3.40 kg., chloralose anaesthesia. Tracing of response of the preganglionically stimulated nictitating membrane. At first arrow, 56 μg./kg. hexamethonium intravenously. At second arrow, 51 μg./kg. pempidine intravenously. Time, 1 min.

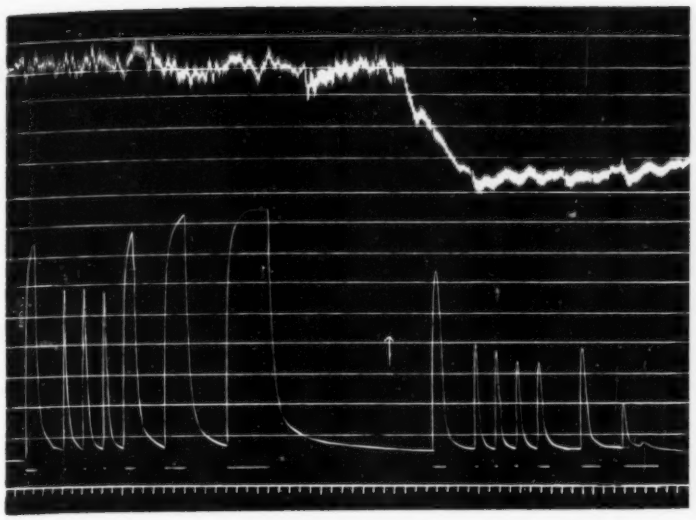


FIG. 2.—Cat, 2.70 kg., thiopentone sodium (10 mg./kg.) followed by chloralose (60 mg./kg.) anaesthesia. Upper tracing, blood pressure; middle tracing, contractions of nictitating membrane stimulated preganglionically for 5, 30, 60, and 120 sec. as indicated. Lowest tracing, time in 30 sec. intervals. At arrow, 0.1 mg./kg. pempidine intravenously. Vertical scale, blood pressure (mm. Hg).

nerve caused a reduced but fully maintained contraction. We have failed to confirm this observation with either mecamylamine or pempidine although various doses and time intervals after injection have been tested. In our experiments with mecamylamine and pempidine, the contractions of the nictitating membrane waned rapidly as with hexamethonium (Fig. 2).

Site of Action.—Pempidine was found to act specifically at the ganglion and not on the nictitating membrane itself, nor on the postganglionic nerve. Thus an intravenous dose of 8 mg./kg. caused complete relaxation of the nictitating membrane stimulated preganglionically whereas there was no diminution of the response to adrenaline injected intravenously nor to stimulation of the postganglionic nerve (Fig. 3).

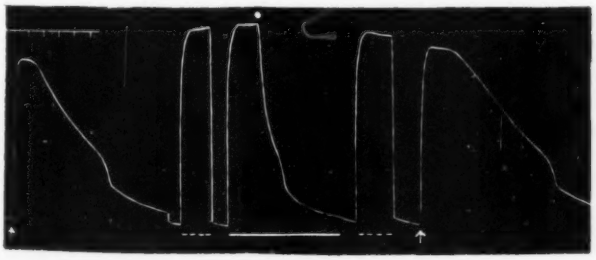


FIG. 3.—Cat, 2.25 kg., chloralose anaesthesia. Contractions of nictitating membrane. At arrows, 100 μg. adrenaline was given intravenously; the solid line indicates preganglionic stimulation at 10/sec.; the broken line indicates postganglionic stimulation at 10/sec.; at the white dot, 8 mg./kg. pempidine was administered intravenously. Time, 30 sec.

Effect on Release of Acetylcholine.—There was evidence that large doses of pempidine reduced the output of acetylcholine from the perfused superior cervical ganglion during preganglionic stimulation. The results of one experiment are shown graphically in Fig. 4. During periods with no stimulation there was no detectable acetylcholine present in the samples (less than 3 ng./ml.). The activity in the first two samples collected during stimulation was equivalent to 13 and 15 ng./ml. respectively (totally 57 and 54 ng.). After the injection of 10 mg. of pempidine into the arterial cannula, the two samples collected during stimulation, which failed to cause a contraction of the nictitating membrane, each contained 10 ng./ml. (totally 32 and 31 ng.). However, this is still a

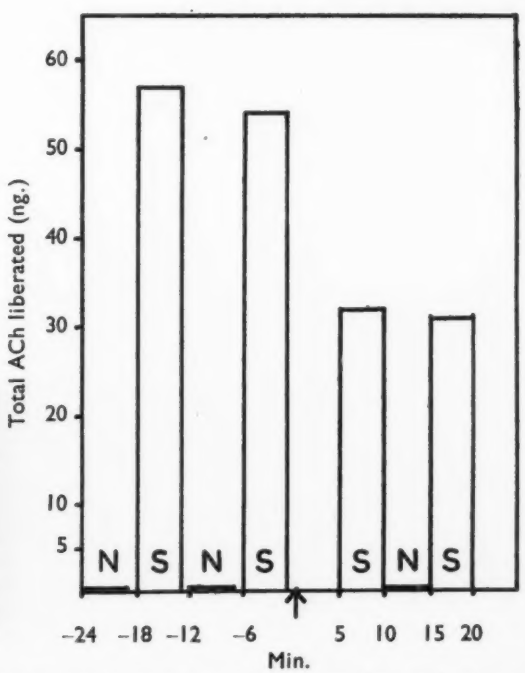


FIG. 4.—Histogram of acetylcholine output from perfused superior cervical ganglion. Ordinates, total output of acetylcholine during 5 min. collection periods. Abscissae, time in min. before and after the injection of 10 mg. pempidine at the arrow. N, no stimulation; S, preganglionic nerve stimulated for 3 min. at 10/sec.

high output of acetylcholine and could not account for the complete block in transmission which was observed.

Effect on the Ciliary Ganglion

Mydriasis in Anaesthetized Cats.—Experiments to compare the effect of pempidine and hexamethonium on pupillary diameter were performed in cats. As with the nictitating membrane preparation it was difficult to obtain graded responses to pempidine due to its prolonged action. From five experiments pempidine had an activity of 2.0 to 4.0 and a duration of action (50% recovery time) of 1.5 to 10.0 times that of hexamethonium. In two other experiments mecamylamine had an activity of 1.5 and a duration of action of 5.0 times that of hexamethonium. There was thus no indication that either of these compounds had a useful differential action on parasympathetic ganglia.

Mydriasis in Mice.—When pempidine or mecamylamine were administered orally to mice, mydriasis was maximal at 10 to 20 min. and persisted for up to 4 hr. Fig. 5 shows the results of two experiments, in the first of which 4 mg./kg. of mecamylamine was compared with 56 mg./kg. of hexamethonium, and in the second experiment the effects of 2 and 4 mg./kg. of pempidine were compared. The large dose of hexamethonium produced only a slight mydriasis, whereas the other two compounds were highly effective in much smaller doses.

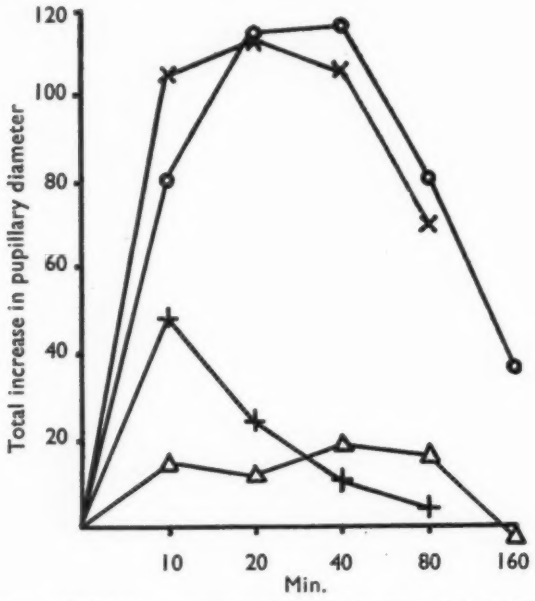


FIG. 5.—Mydriatic responses in groups of 5 mice. ×, 4 mg./kg., +, 2 mg./kg. pempidine orally; O, 4 mg./kg. mecamylamine orally; Δ, 56 mg./kg. hexamethonium orally. Ordinates, total increase in pupillary diameter (arbitrary units); abscissae, time in min. after dosing.

Action on the Cardiovascular System

In vivo *Experiments.*—Doses of 0.05 to 1.0 mg./kg. of pempidine produced a fall in blood pressure which developed more slowly than with hexamethonium and reached a maximum in 3 to 10 min. (Fig. 2). As with hexamethonium the fall was dependent on the initial blood pressure. Thus in one experiment 0.25 mg./kg. produced a fall from 200 to 90 mm. Hg. A second dose had no further effect although when the blood pressure was increased by inducing anoxia with a paralysing dose of decamethonium the same dose of the compound again caused a fall in blood pressure.

It was difficult to produce a fall in blood pressure in anaesthetized cats when the compound was administered orally. However, when injected directly into the duodenum the subsequent fall in blood pressure became maximal at 30 min. In four cats and one dog 0.5 mg./kg. of pempidine, administered in this way, produced falls in blood pressure from 100–160 to 70–100 mm. Hg. In another cat 0.2 mg./kg. produced a fall from 180 to 90 mm. Hg, and in all these experiments the effect was maintained for at least 5 hr.

Up to 10 mg./kg. of pempidine injected intravenously was without effect on the depressor actions of acetylcholine and histamine and, as with other ganglion-blocking compounds, the pressor response to adrenaline was potentiated. The nicotine pressor effect was abolished by a dose of 0.1 mg./kg. and in another experiment a dose of 0.025 mg./kg. caused a 47% reduction in the response produced by 75 μg./kg. of nicotine injected every 15 min. This reduction in the nicotine response was prolonged, 50% recovery occurring only after 2 hr.

In fully atropinized cats, doses up to 64 mg./kg. caused no delayed depressor response, thus indicating the absence of histamine releasing properties. On the contrary when the blood pressure was initially low or had already been depressed to below 80 to 100 mm. Hg by a previous dose of the compound, intravenous doses of 4 mg./kg. or more produced a pressor response which commenced 5 to 10 sec. after injection and having reached a peak within 10 to 60 sec. declined over a period of 5 to 15 min. Fig. 6*a* shows an experiment in which the initial blood pressure was 80 mm. Hg. A dose of 16 mg./kg. of pempidine produced a rise of 50 mm. Hg which then declined

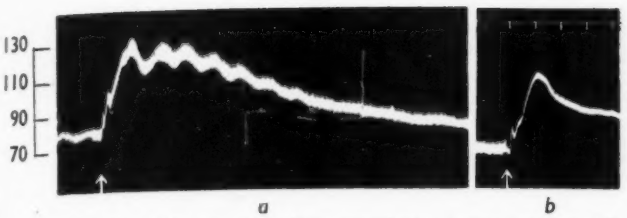


FIG. 6.—Cat, 2.50 kg., chloralose anaesthesia. Blood pressure (*a*), before; (*b*), after acute bilateral adrenalectomy. At arrows, 16 mg./kg. pempidine. Vertical scale, blood pressure (mm. Hg). Time, 1 min.

over a period of 15 min. This pressor effect contrasts with the effect of large doses of mecamylamine (Payne and Rowe, 1957), which invariably caused a further transient fall in blood pressure. In a few experiments the pressor response to large doses of pempidine was preceded by a short-lasting fall in blood pressure similar to that seen with mecamylamine.

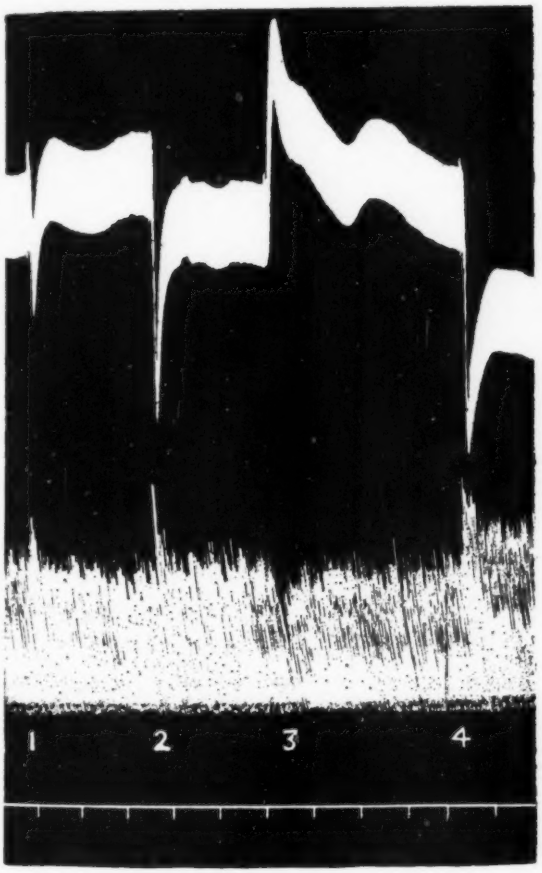


FIG. 7.—Responses of the hind limb of dog perfused with heparinized blood. Upper tracing, arterial pressure; middle tracing, venous outflow. Lowest tracing, time in 1 min. intervals. At **1**, 32 mg. of pempidine; **2**, 1 μg. of acetylcholine; **3**, 1 μg. of adrenaline; **4**, 8 mg. of mecamylamine.

The pressor action of pempidine could not be attributed to the release of adrenaline from the adrenal medulla, since it was very rapid in onset, was not abolished by phenoxybenzamine, nor by bilateral adrenalectomy (Fig. 6*b*). After bilateral vagotomy, and in spinal or pithed preparations, the effect was still present although very slight and evisceration of the animal did not abolish the response.

A dose of 0.4 mg./kg. abolished the bradycardia and depressor effect of peripheral vagal stimulation, although much larger doses did not prevent the effects of an injection of acetylcholine.

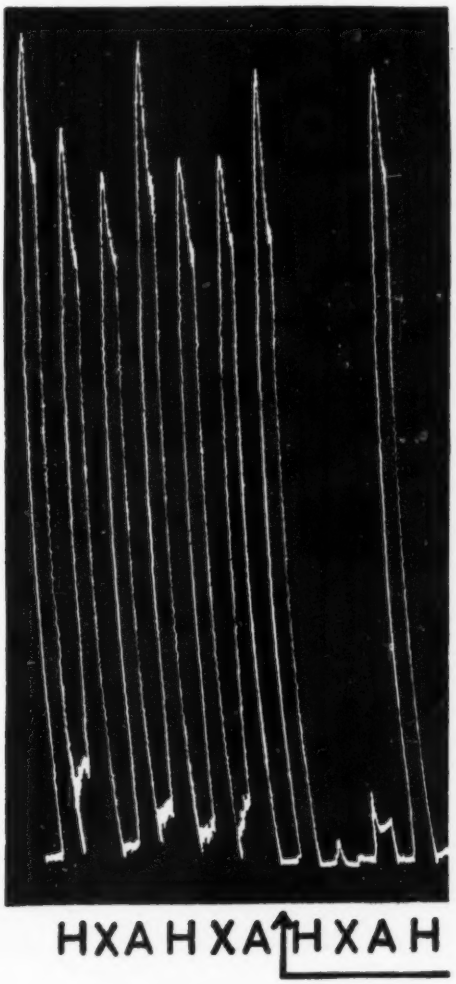


FIG. 8.—Contractions of isolated guinea-pig ileum in a 2 ml. bath. At H, 0.05 μg./ml. histamine; X, 8.0 μg./ml. pempidine; A, 0.25 μg./ml. acetylcholine. The arrow indicates the presence of 0.1 μg./ml. atropine in the bath fluid.

In vitro *Experiments.*—On the isolated rabbit heart about 16 mg. of pempidine injected directly into the aortic cannula was required to arrest the heart for about 30 sec. About 8 mg. of mecamylamine was required to produce a similar effect. Smaller doses of either compound caused a slowing and decrease in amplitude of the beat. These effects have been described for mecamylamine by Bennett *et al.* (1957), using the isolated cat heart which was arrested by 1 mg. of the drug. Spinks and Young (1958) found that a concentration of 0.01 mg./ml. of pempidine in the perfusion fluid caused a decrease in the coronary flow.

Perfused Hind Limb of Dog.—In view of the pressor action seen with large doses of pempidine in *in vivo* experiments, the effect of the compound on the vessels of the hind limb of the dog perfused with heparinized blood was determined. Doses of less than about 8 mg. injected into the arterial cannula were without effect. Larger doses invariably produced vasodilatation (Fig. 7). Vasoconstrictor effects were never observed. Mecamylamine was found to have a considerably greater vasodilator action than pempidine (Fig. 7).

Effects on Isolated Guinea-pig Ileum

In most preparations, pempidine in concentrations up to 800 μg./ml. had little or no stimulant action on the isolated guinea-pig ileum. In other preparations concentrations as low as 8 μg./ml. repeatedly produced a contraction which was abolished by atropine (Fig. 8), potentiated by physostigmine, but unaffected by hexamethonium. In refractory preparations, pempidine selectively blocked nicotine contractions. Fig. 9 shows an experiment in which 0.8 μg./ml. of pempidine was present in the bath for 17.5 min. The response to nicotine was inhibited, leaving contractions to histamine, pilocarpine, and acetylcholine unaffected. After pempidine had been removed from the bath the nicotine response was slow to recover, so that even in *in vitro* preparations the compound has a prolonged action. A positive correlation between concentration and recovery of the nicotine response was observed (Table I).

When the concentration of pempidine was increased, the responses to other agonists were reduced. Thus in an experiment in which 80 μg./ml. of pempidine was present for 14.5 min., the responses to nicotine and histamine were abolished and the responses to pilocarpine and acetylcholine reduced. On removal of the antagonist, the responses to the latter two compounds rapidly returned but those to nicotine and histamine remained depressed for several hours. Spinks and Young (1958) have also reported that

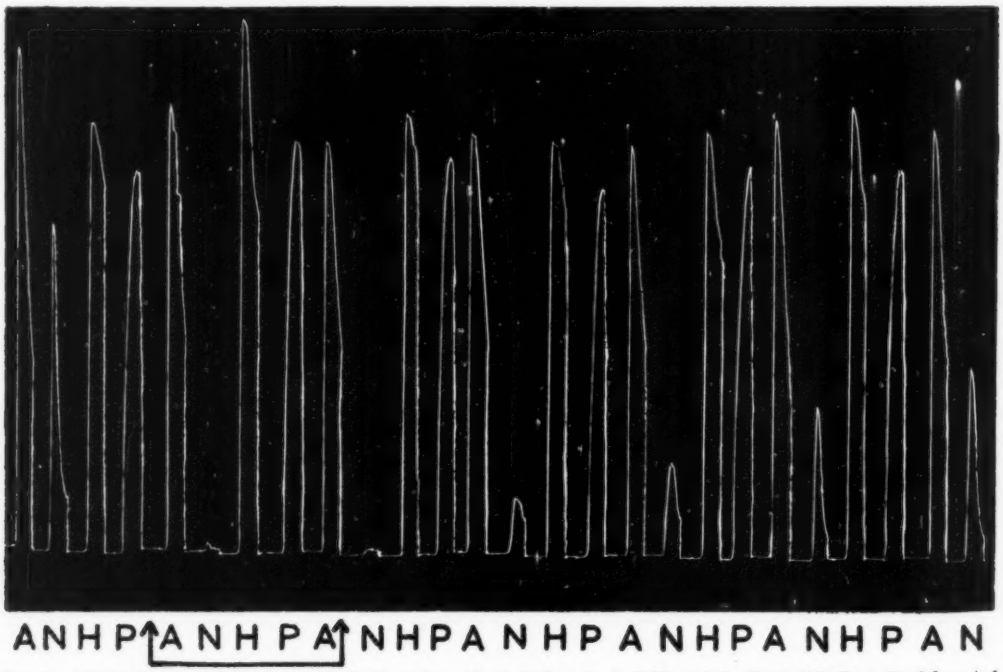


FIG. 9.—Contractions of isolated guinea-pig ileum in a 10 ml. bath. At **A**, 0.05 μg./ml. of acetylcholine; **N**, 5.0 μg./ml. of nicotine; **H**, 0.1 μg./ml. of histamine; **P**, 1.0 μg./ml. of pilocarpine. Between arrows, 0.8 μg./ml. of pempidine was present.

high concentrations of pempidine inhibit responses to 5-hydroxytryptamine.

In Trendelenburg preparations of the isolated guinea-pig ileum, pempidine (1 μg./ml.) inhibited peristaltic waves without preventing contraction of the longitudinal muscle.

Effect of Nicotine on Histamine Responses.—In the experiments in which the duration of action of pempidine on nicotine responses was studied (Table I) nicotine was placed in the bath after

TABLE I

RELATIONSHIP BETWEEN CONCENTRATION OF PEMPIDINE AND TIME FOR 50% RECOVERY OF NICOTINE RESPONSES OF GUINEA-PIG ILEUM

The contact time for pempidine was 14.5 min. Each numeral is the mean of 3 experiments and ± standard deviation is shown.

Conc. μg./ml. ..	0·8	2·6	8·0
Time for 50% recovery (min.) ..	12·5±3·8	58·3±3·6	110·5±48·0

three consecutive histamine responses. Following a nicotine response there was often some reduction in the succeeding one or two responses to histamine and therefore each nicotine response was expressed as % of the immediately preceding histamine response. In some preparations the effect of nicotine on the histamine response was sufficiently great to make the preparation unsuitable for further use, and even small doses of nicotine, which themselves produced no contraction, reduced the succeeding response to histamine. Nicotine was not observed to have this effect on acetylcholine responses.

Effect on Neuromuscular Transmission

Effect on Sciatic Nerve-Tibialis Muscle Preparation in the Cat.—Intravenous doses of 10 to 40 mg./kg. of pempidine were required to block the response of the indirectly stimulated tibialis muscle. The block resembled that due to tubocurarine since tetanic stimulation failed to produce a sustained contraction, was followed by marked potentiation of the twitch tension and neostigmine reversed the block. Pempidine resembled mecamylamine potentiating the effect of tubocurarine and antagonizing the effect of decamethonium (Bennett *et al.*, 1957).

Effect on Rectus Abdominis Muscle of the Frog.—On this preparation pempidine showed no stimulant properties in concentrations up to 0.8 mg./ml. although this concentration inhibited acetylcholine-induced contractures.

Anticholinesterase Activity.—This was determined by the method of Hobbiger (1951) using washed erythrocytes obtained from fresh defibrinated horse blood as a source of true acetylcholinesterase free from pseudo-esterase activity. Under similar conditions, pempidine was found to be about 10^6 times less active than neostigmine in inhibiting this enzyme system. Thus 50% inhibition was produced by a concentration of 3.6×10^{-8} M-neostigmine whereas a concentration of 2.1×10^{-2} M-pempidine was required to produce the same result.

Effect on Nicotine Convulsions in Mice

Several ganglion-blocking compounds were examined for their ability to protect mice from convulsions produced by the intravenous injection of 1 mg./kg. of nicotine base. Table II shows the

TABLE II

THE INHIBITORY EFFECT OF GANGLION-BLOCKING COMPOUNDS ON NICOTINE INDUCED CONVULSIONS IN MICE

Compound	ED50 (mg. cation/kg.)	Comparative Activity Against Nicotine Convulsions		Comparative Activity on Cat Nictitating Membrane	
		Hexamethonium =1	Mecamylamine =1	Hexamethonium =1	Mecamylamine =1
Hexamethonium	1·5	1	0·072	1·0	0·82
Pentolinium ..	0·25	6	0·43	4·0	3·3
Mecamylamine	0·108	13·9	1·0	1·22	1·0
Pempidine ..	0·09	16·7	1·2	1·35	1·11

results obtained using two quaternary ammonium compounds and two non-quaternary compounds. Whereas there is a good correlation within each class with activity obtained on the cat nictitating membrane, between the two classes of compound there is no such correlation, hexamethonium and pentolinium being considerably less effective than mecamylamine and pempidine.

Effects on the Central Nervous System

Effects of Intracerebroventricular Injections in Mice.—The effects produced by pempidine were compared with those produced by mecamylamine and hexamethonium. Animals which received physiological saline alone became motionless for about 1 min. immediately after the injection and then resumed normal activity. Following an injection of 10 μg. of hexamethonium the mice became motionless in a hunched up posture. After about 3 min. generalized tremors were seen in some animals. 50 μg. produced dyspnoea, generalized tremors and convulsions lasting for about ½ to 1 hr. Still higher doses increased the severity and duration of these symptoms. These signs are similar to those observed by Haley and McCormick (1957).

Following an injection of 100 μg. of pempidine the animals became motionless, assuming a hunched up posture. No tremors were observed although after a dose of 200 μg. slight tremors were seen in most of the mice. Mecamylamine had a similar effect in the same doses.

All the mice used in these experiments appeared normal after 24 hr. and in no instance did the non-quaternary compounds produce effects approaching the severity of those seen with hexamethonium.

Effect on Knee-jerk and Flexor Reflexes in the Cat.—In cats anaesthetized with chloralose, pempidine had no effect on the knee-jerk or flexor reflex except in doses which also reduced the twitch tension of the contralateral tibialis muscle stimulated peripherally by the sciatic nerve.

Toxicity

A 5% saline solution of the hydrochloride of pempidine injected intracutaneously in guinea-pigs produced no local reaction whereas 2% and 5% solutions of mecamylamine hydrochloride caused erythema and necrosis at the injection site.

The LD50 in mice of pempidine and of mecamylamine are shown in Table III. Our estimate

TABLE III
ACUTE TOXICITY IN MICE

Compound	Route of Injection	LD50 (mg. cation/kg.)	Oral LD50 / Intravenous LD50
Pempidine ..	Oral	275	
	Subcutaneous	134	6·9
	Intravenous	39·5	
Mecamylamine	Oral	97·5	
	Intravenous	12·9	7·6

of the intravenous toxicity of mecamylamine is higher than that found by Stone *et al.* (1956) and consequently we have obtained a higher oral LD50/intravenous LD50 ratio than these authors. In our experiments the intravenous injections were given at a rate of 1.0 ml./15 to 20 sec. and, as the acute toxicity is related to the speed of injection, this may account for the difference between results. By both oral and intravenous routes pempidine was about one-third as toxic as mecamylamine.

Toxic effects after oral administration of either compound appeared within minutes. In addition to dyspnoea and clonic convulsions, large doses caused generalized tremors and slight ataxia. In rats the acute oral LD50 of pempidine was 470 mg./kg.

When large doses of either compound were administered orally to young rats, inhibition of growth occurred. Table IV shows % inhibition of growth after 10 daily doses. The last column of Table IV shows the calculated dose to produce 50% inhibition of growth.

TABLE IV
EFFECT ON GROWTH RATE OF YOUNG RATS

Compound	Daily Dose (mg. cation/kg.)	% Inhibition of Growth	Deaths	Calculated Dose to Produce 50% Inhibition of Growth (mg. cation/kg.)
Pempidine ..	40	45·3	0/5	48·0
	80	64·0	1/5	
	160	95·6	3/5	
Mecamylamine	15	47·9	0/5	
	30	63·6	1/5	16·5
	60	89·6	4/5	

No abnormalities occurred in the blood pictures of a group of guinea-pigs injected subcutaneously for 4 weeks (5 times/week) with 4 mg./kg. of pempidine. The tissues from groups of rabbits, guinea-pigs, and rats which had received up to 4 weeks' treatment with the compound were kindly examined by Dr. R. Williamson and Professor C. V. Harrison. Guinea-pigs and rabbits which had received 6.4 mg./kg. subcutaneously and intravenously respectively and rats which had received 12.8 mg./kg. orally were examined by Dr. Williamson, who reported: "In all the animals (rabbits, guinea-pigs, and rats) sections of the liver, kidneys, heart, lungs, spleen, pancreas, suprarenals, and bone marrow were examined. In addition sections of the spinal cord were examined in the rabbits and guinea-pigs. At post-mortem examination no gross changes were observed in the organs of any of the animals. Histological examination did not reveal any significant pathological lesion (one rabbit and one rat had mild inflammatory changes in the bronchi). . . . In my opinion (the compound) has not produced any significant histological lesions in any of the organs of the animals examined."

Professor Harrison reported on guinea-pigs and rabbits which had received 12.8 mg./kg. subcutaneously and intravenously respectively and rats which had received 51 mg./kg. orally for 1½ weeks. The tissues examined were: salivary gland, oesophagus, stomach, jejunum, pancreas, liver, gall bladder, lung, spleen, bone marrow, heart muscle, voluntary muscle and included nerves, cervical lymph node, kidney (including pelvis), fallopian tube, ovary, testis, suprarenal,

thyroid, parathyroid, and brain. Professor Harrison concluded that "all tissues examined appeared healthy except for an occasional focus of foreign body reaction in the lungs."

Discussion

Although non-quaternary ammonium ganglion-blocking compounds have been known for some time, the first such compound shown to possess a high specificity of action combined with a high potency was mecamylamine (Stone *et al.*, 1956). The present compound, pempidine, like those described by Koppanyi and Vivino (1946), Norton and Phillips (1953) and Phillips (1955, 1957), is a piperidine derivative and a tertiary amine whereas mecamylamine is a secondary amine and an isocamphane derivative. However, the secondary amine corresponding to pempidine possesses similar activity and the quaternated compound is also a potent (3 times hexamethonium) but short acting ganglion-blocking compound (Corne and Edge, unpublished observation; Spinks and Young, 1958).

Pempidine, although not devoid of other actions, is a selective ganglion-blocking compound and combines a prolonged duration of action with good absorption from the gastro-intestinal tract as can be judged from the low ratio of oral to intravenous toxicity, the rapidity with which mydriasis develops after oral administration of small doses to mice and the effectiveness of the hypotensive properties of the compound when introduced directly into the duodenum of the anaesthetized cat. Furthermore, there is a rapid rise in the blood concentration after oral administration to rats (Reading, personal communication). The weak muscarinic properties which can sometimes be seen on the isolated guinea-pig ileum would tend to counteract the parasympathetic ganglion-blocking activity of the compound and suggests that a reduced incidence of parasympathetic side-effects may be encountered clinically. This stimulant action on the isolated ileum was potentiated by physostigmine and this suggests that the effect may be due not to an acetylcholine-like action but to the liberation of acetylcholine (Renshaw, Green and Ziff, 1938).

The prolonged duration of action of these amines and their chemical dissimilarity to the quaternary ammonium compounds suggests that the two classes of compound may have fundamentally different modes of action. Using mecamylamine, Bennett *et al.* (1957) have described a difference in the type of response obtained from the preganglionically stimulated nictitating membrane, but we have not been able to confirm this observation and can offer no explanation for the difference observed.

Milne, Rowe, Somers, Meuhrcke, and Crawford (1957), and Payne and Rowe (1957), have shown that certain organs selectively store mecamylamine, whilst Payne and Rowe (1957) have further shown that carbon dioxide inhalation increases the plasma level and potentiates the hypotensive and neuromuscular blocking effects of mecamylamine. Furthermore, Zawoiski, Baer, Braunschweig, Paulson, Shermer, and Beyer (1958) have shown that mecamylamine is excreted into the lumen of the stomach and absorbed by the small intestine so that it undergoes a cycle of absorption and excretion which will also contribute to its prolonged action. However, these mechanisms cannot be solely responsible for the long duration of action of these compounds since small doses injected intra-arterially close to the superior cervical ganglion also exert a more prolonged action than is seen with hexamethonium. These compounds may act intracellularly, or, by virtue of their subsequent slow release from the ganglion cell body, they may block the acetylcholine membrane receptors in the same way as has been postulated for the quaternary ammonium compounds. Alternatively, their prolonged action may be due to stronger binding at the membrane receptors themselves.

There is some evidence (Crawford, cited by Payne, 1957) that mecamylamine inhibits the synthesis of acetylcholine. Although the acetylcholine output of the preganglionically stimulated superior cervical ganglion was not abolished by a large dose of pempidine, inhibition of choline-acetylase could contribute to the long duration of action of these compounds by preventing the replenishment of available acetylcholine. However, the perfusion experiments show that pempidine does not appreciably diminish the release of acetylcholine.

Pempidine has a similar pK_a value (10.4) to mecamylamine (11.3) and is also lipoid soluble so that it is likely to have similar distribution and excretion characteristics to mecamylamine. However, there is evidence (Harington, Kincaid-Smith and Milne, 1958) that pempidine exhibits less protein-binding than does mecamylamine and this will undoubtedly modify its distribution and excretion pattern, and hence its duration of action. Blood and urine levels in both rats (Reading, personal communication) and patients have shown that pempidine is excreted more rapidly than mecamylamine and in clinical use pempidine has been

found to have a less prolonged action than mecamylamine (Harington *et al.*, 1958).

The cause of the rise in blood pressure which can be seen with large doses of pempidine remains obscure since it occurs against a background of complete ganglion paralysis and to a very slight extent in preparations in which the central nervous system is completely destroyed. It cannot be attributed to the release of adrenaline from the adrenal medulla since it occurred after adrenalectomy, and Spinks and Young (1958) have shown that small doses inhibit the effects of stimulation of the splanchnic nerve. Lockett (1949) found that the pressor action of piperidine was not greatly affected by adrenalectomy but was considerably reduced by acute total sympathectomy and reversed by dibenamine. It could be that pempidine in large doses overcomes its own ganglion-blocking action and stimulates sympathetic ganglia, but phenoxybenzamine did not inhibit the effect and no evidence of ganglion stimulation was obtained during perfusion of the superior cervical ganglion. Unless there is a species difference the pressor response cannot be accounted for by a direct vasoconstrictor action since vasodilatation was the only response observed during perfusion of the hind limb of the dog. In this preparation, mecamylamine was considerably more potent than pempidine in causing vasodilatation, and this, together with its direct inhibitory action on the heart, could account for the sharp fall in blood pressure which occurs when this compound is injected into a preparation in which autonomic paralysis is already maximal.

The facility with which mecamylamine and pempidine enter the central nervous system is indicated by the occurrence of tremors when large doses are given orally or parenterally and it seems likely that ganglion-blocking compounds, which are readily absorbed from the gastro-intestinal tract, will, for similar reasons, exhibit effects on the central nervous system. However, the lower toxicity and more rapid excretion of pempidine suggest that these symptoms may occur less frequently in patients than with mecamylamine. It appears that mecamylamine and pempidine have intrinsically less effect on the brain than the quaternary compounds, hexamethonium and pentolinium, since when given by intracerebroventricular injection, the former compounds were less effective in causing tremors than were the latter compounds.

Laurence and Stacey (1953) showed that quaternary ammonium ganglion-blocking compounds antagonize nicotine convulsions by inhibiting the peripheral release of adrenaline by nicotine. In our experiments mecamylamine and pempidine were found to be considerably more effective than hexamethonium and pentolinium in this property, and it seemed that this enhanced effect may have been due to inhibition of the central effect of nicotine. However, pempidine was found to have no inhibitory effect on convulsions induced by leptazol or strychnine except in near-toxic doses.

Inhibitory effects of nicotine on histamine induced contractions of the guinea-pig ileum have been previously described by several authors (for references see Ambache and Rocha e Silva, 1951), but the effects we have observed appear to be different from those previously described. Ambache and Rocha e Silva (1951) distinguished two separate blocking actions. With large paralysing doses of nicotine, histamine contractions were reduced only whilst the nicotine was still present, whereas after removal of nicotine from the bath the histamine responses were unaffected despite the persistence of nicotine paralysis. With smaller stimulating doses there was a prolonged residual inhibition of histamine responses and this resembled the Cantoni and Eastman (1946) effect following large doses of other agonists. However, we have found that small stimulating and even non-stimulating doses of nicotine will reduce the effect of succeeding histamine responses whilst having no effect on acetylcholine responses. The effect we have observed is analogous to that recently described by Dale (1958), who found that small stimulating doses of acetylcholine inhibit the response of the guinea-pig ileum to histamine.

We wish to thank Dr. R. Wien for his interest and encouragement, Dr. R. Williamson and Professor C. V. Harrison for the histological examination of tisues, our colleagues, Dr. W. R. Wragg and Mr. G. E. Lee, who devised and synthesized pempidine, and Mr. W. A. Freeman, Mr. A. C. Rasmussen, Mr. P. Reedy, and Mrs. M. Davies for several estimations.

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ANTITHYROID ACTIVITY OF THIOHYDANTOINS

BY

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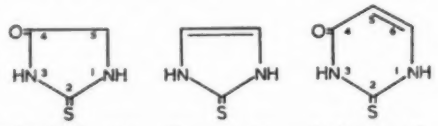
The antithyroid activity of 2-thiohydantoin and some derivatives has been measured in rats, after a single dose and, also, after daily administration for 6 weeks. 2-Thiohydantoin and its 5-alkyl derivatives from 5-methyl to 5-sec-butyl- showed considerable antithyroid activity at a dose of 0.05 m.mole/kg. by mouth, but 5-*n*-hexyl-2-thiohydantoin had insignificant activity at this dose level. The presence of polar groups in the 5-substituent was associated with reduction or complete loss of activity. By the single-dose technique, 2-thiohydantoin was more potent than its 5-alkyl derivatives. The opposite result was obtained by other methods of assessment after 42 days of treatment. Some of the possible factors involved are discussed especially in relation to the difficulty of comparing results obtained by the different methods of assessment. The acute toxicity of 2-thiohydantoin and of its 5-methyl and 5-ethyl derivatives is reported. No toxic effects were found after daily administration of several 2-thiohydantoins at a dose of 50 mg./kg. for 6 weeks.

The biological activity of thiohydantoins, unlike that of hydantoins, has not received much attention in the past. Lewis (1913) reported some acute toxicity studies. Astwood, Bissell, and Hughes (1945), Jackman, Klenk, Fishburn, Tullar, and Archer (1948) and Searle, Lawson, and Morley (1951) included some thiohydantoin derivatives in the large number of compounds which they examined for antithyroid activity. Thiohydantoin is very similar structurally to thiouracil and mercaptoglyoxaline (Fig. 1) and certain of its



2-thiohydantoin 2-mercapto-glyoxaline 2-thiouracil

FIG. 1.

derivatives, like the parent compound, were found to have antithyroid activity. However, no systematic study of this action of thiohydantoins has been reported. 2-Thiohydantoin, sixteen 5-substituted derivatives, 1-methyl-2-thiohydantoin, and thiohydantoic acid, have been tested for antithyroid activity in rats. This activity has been measured in various ways and compared with that of 2-thiouracil. The relationship of structure and activity is discussed with reference to previous work on derivatives of 2-thiouracil and 2-mercaptoglyoxaline.

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METHODS

Preparation of Compounds.—All the compounds tested have been described previously. References to the methods of preparation of 2-thiohydantoins are listed here: 5-carboxymethyl- (Johnson and Guest, 1912), 5-carbamoylmethyl- and 5-indolylmethyl- (Swan, 1952), 5-*p*-hydroxybenzyl- (Taschner, 1951), 1-methyl- and 5-(4′-aminobutyl)- (Elmore and Ogle, 1957), 5-methylsulphonylethyl (Elmore, Ogle, and Toseland, 1956), and the remainder were prepared by the method of Jackman *et al.* (1948). Thiohydantoic acid (*N*-thiocarbamoylglycine) was prepared by the method of Elmore, Toseland, and Tyrrell (1955). All compounds were chromatographically pure (Elmore and Ogle, 1957).

Suppression of ^{131}I Uptake.—This was measured in young rats (about 6 weeks old) following the technique described by McGinty, Rawson, Fluharty, Wilson, Riddell, and Yee (1948). A similar method was used by Searle, Lawson, and Hemmings (1950). Each substance was given in aqueous solution by stomach tube, in a dose of 0.05 m.mole/kg. body weight. All were relatively insoluble and some were given as solutions of the Na salts. Groups of 5 to 7 rats were used to test each substance and as controls, which received water. One hour after dosing, all rats were given an intraperitoneal injection of radioiodide

(approximately 3 μc. of ^{131}I in 1.0 ml. H_2O, without added carrier). The rats were killed with chloroform after a further 4 hr. The thyroid glands were removed immediately and dissolved overnight in 2.0 ml. of 2% NaOH in an incubator at 37°. The volumes were then made up to 10 ml. and counted in a Veall (1948) liquid counter. The ^{131}I content was expressed as % of the injected dose.

Antithyroid Activity after Prolonged Administration of Test Substances.—Young rats (about 6 weeks old) were matched for weight and kept in individual cages. They were allowed water and commercial cube diet *ad libitum*. Thyroid activity was assessed by three criteria, apart from ^{131}I uptake described above.

Weight Gain.—Body weight was measured every third day, after voidance of urine and faeces.

Thyroid Weight.—The thyroid glands were dissected out after death and weighed on microscope slides.

Thyroid Histology. — Thyroid hyperplasia was assessed histologically by a mid-section through one lobe. Assessment was performed using the same criteria and scoring system as used by Astwood (1943).

Results

Antithyroid Activity Following Single Dose of Drug.—The mean depression of ^{131}I content of the thyroid glands for each substance tested is expressed as % of the control glands (Table I). 2-Thiohydantoin is very active, but thiohydantoic acid, which is prepared from it by mild alkaline hydrolysis, is without activity. 1-Methyl-2-thiohydantoin is similar to the parent compound. All the other compounds are 5-substituted thiohydantoins. High potency remains with ascending 5-alkyl substitution, until there is a sudden loss of activity with 5-*n*-hexyl thiohydantoin. Ring substitution, illustrated by the 5-benzyl derivative, still gives high activity, but this disappears with 5-*p*-hydroxy-benzyl derivative. This, and succeeding compounds in Table I, show low activity or are inactive. They contain polar groups in the side-chain. 5-*p*-Hydroxy-benzyl-2-thiohydantoin is still inactive when given by intraperitoneal injection, suggesting that inactivity is not due to lack of absorption from the intestine.

Increasing the dose shows that some slight antithyroid activity of a thiohydantoin containing a polar substituent can be demonstrated. 5-*p*-Hydroxy-benzyl-2-thiohydantoin produces 48% depression of 4 hr. ^{131}I uptake, when the dose is increased tenfold, namely to 0.5 m.mole/kg.

Comparative Antithyroid Activity by Single Dose Technique. — Dose/response relationships for several thiohydantoins were studied and compared with that for 2-thiouracil. At least five doses were used for each compound and % depression of 4 hr. ^{131}I uptake calculated by comparison with the mean uptake in a control group of rats. Regression slopes of the dose/response curves were calculated and relative potency of the substances was compared at the level of 70% depression of ^{131}I uptake (Table II and Figs. 2 and 3). The dose/response curves were reduced to linearity within the ranges of observations, by using logarithmic scales. The horizontal logarithmic scale is $\log_{10}$ (dose in mg.) and the vertical logarithmic scale is $\log_{10}\left(1/1-\frac{\%\text{ depression}}{100}\right)$, or in terms of observation, $\log_{10}$ (mean control uptake/mean trial uptake). Regression slopes were then calculated by least squares.

TABLE I

ANTITHYROID ACTIVITY OF VARIOUS THIOHYDANTOINS IN RATS AS TESTED BY A SINGLE ORAL DOSE OF 0·05 M.MOLE/KG.

The significance of differences in ^{131}I uptake between treated and control animals was calculated using Student's *t* test. The level of significance is indicated in the second column of the table, as follows: $^{**}P<0{\cdot}01$; $^{*}0{\cdot}05>P>0{\cdot}01$; N.S. $P>0{\cdot}05$.

Compound	Mean Depression of ^{131}I Content of Thyroid as % of Control Group	No. of Rats Treated	No. of Controls
2-Thiohydantoin	88**	5	5
Thiohydantoic acid ..	−26 N.S.	6	6
1-Methyl- ,, ..	79**	5	5
5-Methyl- ,, ..	82**	5	5
5-Ethyl- ,, ..	80**	6	5
5-*n*-Propyl- ,, ..	75**	6	5
5-*iso*-Propyl- ,, ..	79**	5	5
5-*n*-Butyl- ,, ..	82**	6	6
5-*iso*-Butyl- ,, ..	86**	6	6
5-*sec*-Butyl- ,, ..	86**	5	5
5-*n*-Hexyl- ,, ..	18 N.S.	6	6
5-Benzyl- ,, ..	90**	6	6
5-*p*-Hydroxybenzyl- ,, ..	−18 N.S.	7	5
5-Carbamoylmethyl- ,, ..	3 ,,	7	6
5-Carboxymethyl- ,, ..	−24 ,,	6	5
5-Methylthioethyl- ,, ..	31*	5	5
5-Methylsulphonyl-ethyl- ,, ..	−24 N.S.	6	6
Indolylmethyl- ,, ..	33*	7	7
5-(4′-Aminobutyl)-hydrochloride ,, ..	18 N.S.	6	7

TABLE II

REGRESSION SLOPES OF DOSE/RESPONSE CURVES FOR THIOURACIL AND VARIOUS THIOHYDANTOINS

Compound	Regression Slope (Units in Mean Log Response/Unit of Log Dose) ± S.E.		Common Slope	Dose in mg. for 70% Depression of ^{131}I Uptake (Accurate to −30, +50%, See Text)
2-Thiouracil ..	0·764	0·080	0·735	0·93
2-Thiohydantoin ..	0·741	0·072		1·20
5-Benzyl- ,, ..	0·699	0·084		2·10
5-Methyl- ,, ..	0·457	0·115	0·455	3·00
5-*n*-Propyl- ,, ..	0·452	0·068		2·70
5-*iso*-Butyl- ,, ..	0·619	0·085		3·00
1-Methyl- ,, ..	0·351	0·080		1·50

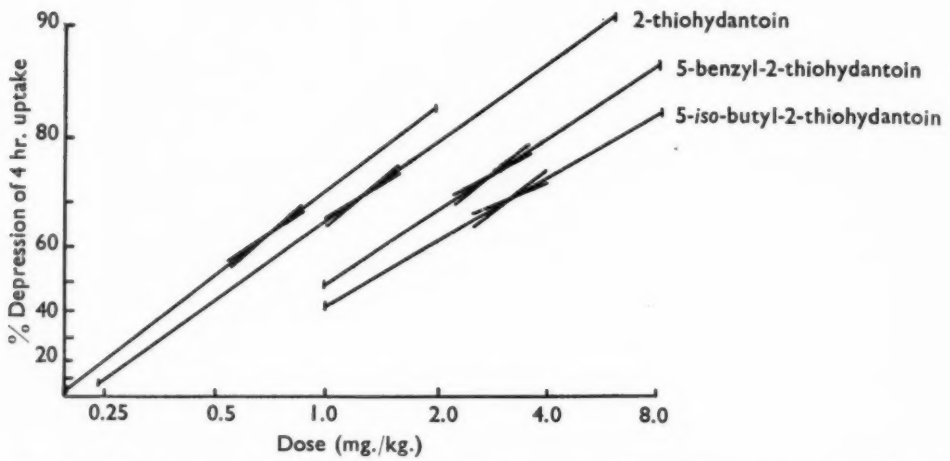

FIG. 2.—Calculated regression lines from dose/response curves for 2-thiouracil, 2-thiohydantoin, 5-benzyl-2-thiohydantoin, and 5-*iso*-butyl-2-thiohydantoin. Horizontal logarithmic scale is $\log_{10}$ (dose in mg.). Vertical logarithmic scale is $\log_{10}$ (mean control uptake/mean trial uptake). ±S.E. of regression line is indicated for each substance. The left-hand regression line refers to 2-thiouracil.

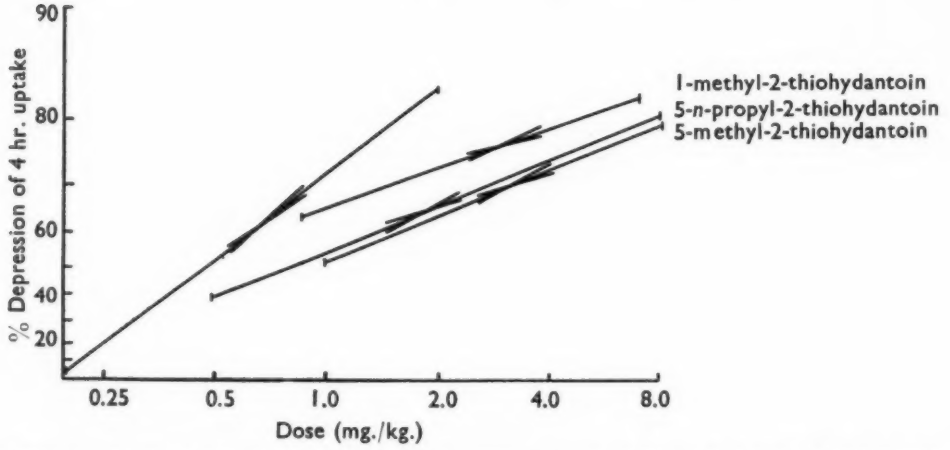

FIG. 3.—Calculated regression lines from dose/response curves for 2-thiouracil, 1-methyl-2-thiohydantoin, 5-*n*-propyl-2-thiohydantoin, and 5-methyl-2-thiohydantoin. Horizontal logarithmic scale is $\log_{10}$ (dose in mg.). Vertical logarithmic scale is $\log_{10}$ (mean control uptake/mean trial uptake). ±S.E. of regression line is indicated for each substance. The left-hand regression line refers to 2-thiouracil.

An analysis of variance, corresponding to a goodness of fit test and a test of parallelism for the regression lines, is shown in Table III. The means of groups of rats given the same dose did not deviate from the regression line more than would be expected from the within group variations; there was thus no indication either of non-linearity of regression or of some uncontrolled experimental error affecting all rats in a dose group, and the deviations gave no indication of statistical significance ($F=0.89$).

The regression lines deviated significantly from a common regression slope ($F=3.41$). Thus relative potency cannot be compared from these

TABLE III

ANALYSIS OF VARIANCE TO TEST FOR A COMMON REGRESSION SLOPE OF DOSE/RESPONSE CURVES AND GOODNESS OF FIT OF REGRESSION LINES

The double asterisk indicates significant at the 1% level.

	Degrees of Freedom	Mean Square	F Criteria
Deviations of regression lines from parallelism	6	1,240	— F criterion = 3·41**
Deviations of dose level group means from regression lines ..	21	287	— F criterion = 0·89 N.S.
Variations within dose level groups	220	364	

slopes, apart from comparison at a given % depression. However, common regression slopes could be ascribed to two groups of substances, as within these groups there was no significant deviation from parallelism. Relative potency can be assessed without qualification within the group 2-thiouracil, 2-thiohydantoin, and 5-benzyl-2-thiohydantoin, and between 5-methyl-2-thiohydantoin and 5-*n*-propyl-2-thiohydantoin, but not between these groups or the other two thiohydantoins tested (see Table II). 2-Thiouracil and 2-thiohydantoin have almost equal potency, and the latter is nearly twice as potent as its 5-benzyl derivative. Interpretation of the flatter slopes of 5-methyl, 5-*n*-propyl-, 5-*iso*-butyl-2-thiohydantoin and 1-methyl-2-thiohydantoin is difficult and is discussed later. All the substances are compared at one particular % depression. It is difficult to give precise standard errors for the doses giving 70% depression, as the mean control uptake for each dose/response curve is based on observations from 7 to 10 rats. Unfortunately, there is significant variation in controls measured at different times, probably due to variations in iodine content of diet, but limits of error probably range from −30% to +50%. When the substances are compared in this way, 2-thiohydantoin is the most potent, and substitution of methyl, *n*-propyl or *iso*-butyl in the 5-position reduces activity about threefold.

Duration of Activity.—The durations of action of 2-thiohydantoin and 5-methyl-2-thiohydantoin are compared with 2-thiouracil (Table IV). 2-Thiohydantoin and its 5-methyl derivative produced greater depression of iodide uptake than 2-thiouracil, when uptake was measured from 1 to 5 hr. after the dose. These thiohydantoins were calculated to be more potent than 2-thiouracil from the dose/response curves. However, when the interval between dosing and giving ^{131}I was lengthened to 4, 8 and 12 hr. this effect of the thiohydantoins became less than 2-thiouracil. This is suggestive evidence that the duration of action of these thiohydantoins was shorter than 2-thiouracil.

Effects of Prolonged Administration.—The effect of various thiohydantoins given daily for 42 days was studied by observing changes in growth rate, thyroid weight, histological changes in thyroid and 24 hr. ^{131}I uptake (Table V). 2-Thiohydantoin and four 5-alkyl derivatives were tested. The other three compounds, 5-benzyl, 5-methylthioethyl- and 5-carboxymethyl-2-thiohydantoin,

TABLE IV

THE DEPRESSION OF ^{131}I UPTAKE PRODUCED BY 2-THIOHYDANTOIN, 5-METHYL-2-THIOHYDANTOIN, AND 2-THIOURACIL. ^{131}I GIVEN 1 HR., 4 HR., 8 HR., AND 12 HR. AFTER THE DRUG AND UPTAKE MEASURED 4 HR. LATER

† Indicates a dose estimated to produce equal depression of ^{131}I uptake at 1 hr.

Compound	Oral Dose† (mg./kg.)	% Depression of ^{131}I Uptake 1 to 5 hr. after Dose	% Depression of ^{131}I Uptake 4 to 8 hr. after Dose	% Depression of ^{131}I Uptake 8 to 12 hr. after Dose	% Depression of ^{131}I Uptake 12 to 16 hr. after Dose
2-Thiohydantoin ..	0·3	88	85	48	21
5-Methyl- ,, ..	0·4	82	73	42	15
2-Thiouracil	0·1	72	67	53	35

TABLE V

EFFECT OF VARIOUS THIOHYDANTOINS ON GROWTH RATE, THYROID WEIGHT AND HISTOLOGY AND 24 HR. ^{131}I UPTAKE IN RATS GIVEN EITHER 50 MG./KG. OR 10 MG./KG. DAILY BY SUBCUTANEOUS INJECTION FOR 42 DAYS

The significance of differences in daily weight gain, thyroid weight and ^{131}I uptake between treated and control animals was calculated using Student's *t* test. The level of significance is indicated as in Table I.

Compound	No. of Rats Dosed		Mean Daily Weight Gain as % of Mean Daily Weight Gain of 8 Control Rats		Mean Increase in Thyroid Weight Compared with Mean Thyroid Weight (mg./100 g./Rat) of 8 Control Rats		Histological Changes of Hyperplasia (2 Rats in Each Group) Classified According to Astwood		Mean Depression (2 Rats in Each Group) of 24 hr. ^{131}I Content of Thyroid as % of Mean of 4 Control Rats	
	50 mg./kg.	10 mg./kg.	50 mg./kg.	10 mg./kg.	50 mg./kg.	10 mg./kg.	50 mg./kg.	10 mg./kg.	50 mg./kg.	10 mg./kg.
2-Thiohydantoin ..	4	4	95 N.S.	96 N.S.	3·4*	2·1*	+++	++	72**	68**
5-Methyl- ,, ..	4	4	76*	93 ,,	3·2**	0·8*	+++	+	63**	58**
5-*n*-Propyl- ,, ..	4	4	86 N.S.	100 ,,	9·0**	2·0**	++++	++	69**	37*
5-*iso*-Propyl- ,, ..	4	4	71**	103 ,,	19·8**	7·8**	++++	+++	86**	45*
5-*iso*-Butyl- ,, ..	4	4	84 N.S.	105 ,,	8·0**	1·6*	++++	+	36**	18 N.S.
5-Benzyl- ,, ..	4	4	56**	89 ,,	27·2**	8·2**	++++	+++	83**	64**
5-Methylthioethyl- ,, ..	4	4	97 N.S.	99 ,,	1·5*	1·0*	+	+	47*	35*
5-Carboxymethyl- ,, ..	4	4	95 ,,	108 ,,	0·0 N.S.	0·1 N.S.	−	−	−3 N.S.	−21 N.S

were chosen to represent an aromatic derivative, and polar compounds. The substances were given by subcutaneous injection at two dose levels, 10 mg./kg. and 50 mg./kg. The smaller of these doses was approximately twice the dose of 0.05 m.mole/kg. used in the single-dose technique. Control rats were given distilled H_2O subcutaneously. Depression of daily weight gain was only found with the 5-methyl, 5-*iso*-propyl, and 5-benzyl derivatives at the level of 50 mg./kg. and this was associated with changes in thyroid weight, histology, and 24 hr. ^{131}I uptake. As judged by these criteria, each substance produced uniform and consistent effects. The one striking exception was 2-thiohydantoin, which had been found, from the dose/response curves to single injections, to be the most potent of the thiohydantoins tested. After 6 weeks of administration there was no depression of weight gain, and only moderate increase of thyroid weight, compared with its 5-methyl *iso*-propyl and benzyl derivatives. However, it still produced pronounced depression of 24 hr. ^{131}I uptake at the end of the 42-day period of administration. The degree of histological change produced by 2-thiohydantoin seemed to correlate with increase of thyroid weight, as it appeared to do for all other compounds tested. Also striking were the differences in effects produced by the two propyl derivatives. 5-Methylthioethyl-2-thiohydantoin was weakly active when tested by the single dose technique and remained so with this method. The compound with the polar carboxyl group, 5-carboxymethyl-2-thiohydantoin, had no activity by either method.

Apart from the effects described above, all the rats remained well and active during administration of these compounds. Liver, kidneys, adrenal glands and testes showed no change in gross weight and no histological changes compared with those from control rats at the end of the 6 weeks period.

Acute Toxicity. — The acute toxicity was measured by LD50 values for three thiohydantoins given by stomach tube as suspensions in 1% methyl cellulose (Table VI). These toxicity studies were followed for 72 hr., but all deaths occurred within 12 hr. Because of the extreme insolubility of these compounds, LD50 values for injection routes could not be measured. Doses of 200 mg./kg., which were the highest that could be conveniently injected subcutaneously in solution, produced no deaths.

TABLE VI

ACUTE TOXICITY OF THIOHYDANTOINS IN YOUNG RATS ABOUT 6 WEEKS OLD

LD50 values (mg./kg. body weight).

Compound	Dose Given Orally as Suspension in 1% Methyl Cellulose
2-Thiohydantoin ..	420
5-Methyl- ,, ..	1,050
5-Propyl- ,, ..	980

DISCUSSION

Several different methods are available for the assessment of antithyroid activity, and for the study of the relation between structure and activity. Such methods fall roughly into two groups. The substance to be tested is given by mouth, or by injection, or in the diet for some days, and antithyroid activity is assessed by the degree of hyperplasia on histological examination (Astwood, 1943), increase in thyroid weight (Astwood *et al.*, 1945 ; Jensen and Kjerulf-Jensen, 1945), decrease in thyroidal iodine concentration (Bywater, McGinty, and Jenesel, 1945). A combination of these measurements has also been used (Astwood *et al.*, 1945 ; Seifter and Ehrich, 1948). Alternatively, antithyroid activity is measured after a single dose of the test substance, using uptake of radioiodine (Larson, Keating, Peacock, and Rawson, 1945). Many compounds were tested in this way by McGinty *et al.* (1948) and later by Searle *et al.* (1950). These observers used experimental animals, and thyroidal radioiodide was measured on dissected thyroids. Stanley and Astwood (1947), using *in vivo* counting and single-dose technique, have assayed drugs for antithyroid activity in man. However, there has been little work on the correlation of results obtained using single or repeated doses and there is a general assumption that comparable results may be obtained by either method (Searle *et al.*, 1950). The results of the present investigation on thiohydantoins do not support this assumption. Using the single-dose technique, dose/response curves indicate that 2-thiohydantoin is almost equipotent with 2-thiouracil, and that 5-methyl, 5-propyl, 5-butyl and 5-benzyl derivatives of 2-thiohydantoin are less active than the parent substance. On the other hand, after daily administration and on assessing the response by increase in thyroid weight and hyperplasia histologically, 2-thiohydantoin appears to be much less active than these derivatives. These results resemble those found by Astwood *et al.* (1945) for 6-substituted derivatives of 2-thiouracil. Propyl and benzyl derivatives were about ten times as potent, and among the alkyl substituted compounds, peak activity was found with the propyl-derivative.

It was also found that activity was greatly diminished in 6-*n*-hexyl-2-thiouracil, and it is of interest that 5-*n*-hexyl-2-thiohydantoin has no activity on acute testing in the present study. This striking relationship between activity and number of carbon atoms on the side-chain of 6-substituted thiouracils was confirmed by McGinty and Bywater (1945a and b), Vanderlann and Bissell (1946) and Anderson, Halverstadt, Miller, and Roblin (1945). The relationship for alkyl substitution in the 5-position of 2-thiouracil is not so apparent (Astwood *et al.*, 1945 ; Anderson *et al.*, 1945).

Much work has also been done with derivatives of 2-mercaptoglyoxaline, which is structurally very similar to 2-thiohydantoin. Searle *et al.* (1950), using the single-dose technique, did not find any increase of activity with 4-alkyl substitution on 2-mercaptoglyoxaline, and they compared this with the results obtained by Astwood *et al.* (1945) on chronic testing. Searle *et al.* (1950) do not appear to have studied dose/response curves with all their 4-alkyl derivatives. It is possible that varying rates of metabolism or excretion may account for differences found between single-dose testing and more chronic administration of compounds. However, no great difference was found between 2-thiohydantoin and its 5-methyl derivative when the rate of return of thyroid activity to normal was measured, and the activity of both of these was of shorter duration than that of 2-thiouracil. Lawson, Rimington, and Searle (1951) noted that the duration of action of 2-mercaptoglyoxalines was apparently less than that of 2-thiouracil. This problem of destruction and excretion requires further study. Jackman *et al.* (1948) studied some 4-substituted mercaptoglyoxalines and 5-substituted thiohydantoins, but related their activity to 2-thiouracil and not to the parent compounds. In agreement with the present results, they found no change in activity with varying numbers of carbon atoms in the 5-alkyl side chain of 2-thiohydantoins. Activity is severely diminished by the presence of a polar substituent at $C_{(5)}$ and in the 2-thiohydantoin ring. This is clearly illustrated by a comparison of the antithyroid properties of 5-benzyl and 5-*p*-hydroxybenzyl-2-thiohydantoin. The largest effect arises from groups such as $-NH_3^+$ $-CO.O^-$ on the one hand and $-CO.NH_2$ $-OH$ on the other. Compounds containing any of these substituents might be bound by plasma proteins, the former by electrovalent linkages and the latter by hydrogen-bonds. Such binding would prevent the drug from reaching the thyroid in a significant concentration. A group such as $-SCH_3$ could be bound rather weakly by an ion-dipole interaction, and it is noticeable that 5-methylthioethyl-2-thiohydantoin has a weak but significant antithyroid activity. Alternatively, interaction between drug and plasma proteins may not be important, and the result may be a true representation of activity within the thyroid. Whatever the explanation may be, a similar correlation between chemical structure and antithyroid properties has been observed with 6-substituted-2-thiouracils (Astwood, 1943 ; Astwood *et al.*, 1945 ; McGinty and Bywater, 1945a and b ; Vanderlann and Bissell, 1946 ; Miller, Dessert, and Anderson, 1948 ; Horner, Kimmig, and Schreiner, 1952), and substituted 2-mercaptoglyoxalines (Astwood, 1943 ; Astwood *et al.*, 1945 ; Searle *et al.*, 1950, 1951).

All the thiohydantoins tested in the present work contain the thioureido group, and this is also common to thiouracils and mercaptoglyoxalines, which have been studied by others. The mechanism of action of compounds containing this group is still not certain, but it is likely that some enzyme system catalysing oxidation of iodide to iodine in the thyroid is blocked (Astwood, 1954). It is therefore disturbing to find significant deviations from parallelism of dose/response curves for different thiohydantoins. The flatter slopes for 5-*n*-propyl, 5-methyl, 5-*iso*-butyl and 1-methyl-2-thiohydantoin may be due to the observations being taken higher up the shoulder of a response curve with a lower limiting % depression. This can occur with enzyme inhibitors of the partially competitive type (Dixon and Webb, 1958). Similar behaviour has also been noted by Astwood (1945) for 2-thiouracil and its derivatives, and he concluded that quantitative comparisons could only be made at some selected level of response. Similar treatment is required for the thiohydantoins and they have been compared here at the level of 70% depression of ^{131}I uptake. The central portion of the dose/response curve using this method approximates to a straight line. Larson *et al.* (1945) and Searle *et al.* (1950) suggest that comparison of potency should be made between the limits of 20% and 80%. Differences of slope for dose/response curves may be a reflexion of differences of inhibitor constant for the various drugs and the enzyme concerned.

The acute toxicity of 2-thiohydantoin was much greater than either its 5-methyl or 5-ethyl derivative. This confirms the toxicity studies on 2-thiohydantoin and its 5-methyl derivative in rabbits reported by Lewis (1913). No toxic effects

were noted from any of the thiohydantoins studied after daily subcutaneous administration of 50 mg./kg. for six weeks. Diminished growth rate, which occurred with a few 2-thiohydantoins, correlated with degree of antithyroid activity, and this delayed growth is probably due to considerable reduction in thyroxine secretion as suggested by Lawson *et al.* (1951). Convulsions, which were reported by Searle *et al.* (1951) to occur with administration of 1-benzoyl-2-thiohydantoin, at a lower daily dose than used in this work, were not observed with any thiohydantoin given.

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PHARMACOLOGICAL ACTIVITY OF 5-PHENYL-1 : 3 : 4-THIADIAZOLE (L 1538), 2-AMINO-5-PHENYL-1 : 3 : 4-THIADIAZOLE (L 1460) AND 2-AMINO-5-(2′-THIENYL)-1 : 3 : 4-THIADIAZOLE (L 1458)

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The pharmacological actions of 5-phenyl-1 : 3 : 4-thiadiazole (L 1538), 2-amino-5-phenyl-1 : 3 : 4-thiadiazole (L 1460) and 2-amino-5-(2′-thienyl)-1 : 3 : 4-thiadiazole (L 1458) have been studied and compared with those of other muscular relaxant drugs. The three compounds have paralysing effects in mice, rats, cats, and dogs. They block the tonic component of maximal electroshock seizures and protect against strychnine, though not against leptazol-induced convulsions in mice. Like other centrally acting paralysing drugs, they depress spinal polysynaptic transmission in doses which leave monosynaptic transmission relatively unaffected. The rigidity of the decerebrate cat is reduced but not always abolished. The compounds do not induce synchronization in epidural electroencephalogram recordings. In doses not greatly affecting muscular tone and spontaneous activity, they prolong the hypnotic effects of pentobarbitone and other barbiturates in mice. Autonomic functions are only slightly affected. The similar actions of substituted thiadiazoles and 2-amino-benzothiazoles confirm a previous hypothesis that pharmacological equivalence may result either from condensing heterocyclic nuclei with aromatic nuclei or from introducing aryl substituents into them.

In a previous paper (Maffii, Testa, and Ettorre, 1958) the synthesis and depressant activity on the central nervous system of a series of substituted 1:3:4-thiadiazoles were described. A possible analogy, from the pharmacological point of view, with substituted benzothiazoles studied by Domino, Unna, and Kerwin (1952) was suggested.

We now report further studies of three of the most interesting compounds in the series of 1:3:4-thiadiazoles: 5-phenyl-1:3:4-thiadiazole (L 1538), 2-amino-5-phenyl-1:3:4-thiadiazole (L 1460), and 2-amino-5-(2′-thienyl)-1:3:4-thiadiazole (L 1458).

MATERIALS AND METHODS

CF-1 mice, both male and female, weighing 20 to 25 g., and CF-Wistar rats weighing 120 to 180 g. were used. Experiments were also carried out on 14 cats and 12 mongrel dogs.

Because of the low solubility of the three compounds, 20% propyleneglycol was used as a solvent for intravenous administration; for other routes suspensions in 5% aqueous gum acacia were used. These were administered orally by stomach tube, except to dogs, which received gelatin capsules.

The anticonvulsant action of L 1538, L 1460, and L 1458 was tested in mice and rats, by intraperitoneal injection, against maximal electroshock seizures and leptazol and strychnine-induced convulsions, as previously described (Maffii *et al.*, 1958).

The effects of L 1538, L 1460, and L 1458 on spinal reflexes were tested using the techniques of Berger (1949). The knee jerk was elicited in cats anaesthetized with pentobarbitone, or in spinal cats, by mechanical tapping of the patellar tendon at a rate of 1/sec. The flexor reflex was elicited by electrical stimulation of the central stump of the posterior tibial nerve. Rectangular pulses of 15 V. and of 0.5 msec. duration delivered at a rate of 400/sec. for a period of 0.1 sec. were used as stimuli. Contractions of the tibialis anterior muscle were recorded.

The effect on decerebrate rigidity was studied in cats in which the brain stem had been sectioned at the level of the superior colliculi and the brain destroyed.

Electromyograms from decerebrate cats were recorded with an electroencephalograph. The electrodes were implanted in the skin over the hyperactive muscles.

Electroencephalograms were recorded from cats. The animals were anaesthetized with ether while the operative procedure was being carried out. The

trachea was cannulated for artificial respiration. The calvarium was exposed and gramophone needles were placed in the bone with their tips in contact with the dura. Placements were made in the frontal, parietal, and occipital regions on the right side and in the parietal or frontal region on the left side. An indifferent electrode was placed in the soft palate. Animals were then curarized. In some experiments the blood pressure by carotid cannulation was also recorded. All experiments were started more than 1½ hr. after the ether had been withdrawn.

Other actions of L 1460 and L 1458 were studied in cats and dogs. Respiratory movements were recorded by tambour connected to the tracheal cannula, and the blood pressure by a mercury manometer from the carotid artery. The peripheral stump of the vagus nerve was stimulated by rectangular pulses (0.5 msec., 10 V., 300/sec., for 0.5 sec.). Carotid occlusion was performed manually using a haemostat. The electrocardiogram was recorded by needle electrodes placed in each foreleg.

Potentiating action on barbiturate-induced hypnosis was tested by injecting the compounds intraperitoneally 20 min. before the selected barbiturate was administered intramuscularly. The duration of loss of righting reflex was measured. The median sleeping time was determined by the graphical method of Litchfield (1949).

Antipyretic action was tested in albino rabbits of both sexes weighing between 2 and 3.5 kg. Rectal thermocouples were used and galvanometer readings were taken half-hourly over a period of 2 hr. before and 5 hr. after intravenous injection of 1 ml./kg. of saline containing pyrogen from *Pseudomonas aeruginosa*. The thiadiazoles were given intraperitoneally 2 hr. after the pyrogen.

To obtain LD50 values and other quantal estimates, the method of Litchfield and Wilcoxon (1949) was followed.

Results

Physical and Chemical Properties

L 1538 crystallized in light-brown, brilliant, odourless platelets with slightly bitter taste. It melted at 36 to 38°. It was almost insoluble in water, but soluble in most organic solvents. L 1460 was an odourless, pink-white, crystalline powder. It melted at 226 to 227° and was very sparingly soluble in water (0.13 g./l. at 38°). L 1458 was first synthesized in our chemical department in 1955 (Maffii *et al.*, 1958). It was a white-yellow crystalline powder. It melted at 243 to 244° and was very sparingly soluble in water (0.23 g./l. at 38°).

Effects in Mice and Rats

A dose of 10 mg./kg. of L 1538, intraperitoneally, in mice had, after a latency of 5 to 10 min., a slight sedative effect: spontaneous activity was slightly decreased as well as reactivity to changes in the environment (cage). After 30 mg./kg., the sedative effect was more pronounced and slight ataxia and impairment of the righting reflex appeared. After 100 mg./kg. spontaneous activity was greatly reduced as well as reactivity to touch and to transfer from cage. The righting reflex was markedly impaired and muscular tone greatly reduced. A slight decrease in corneal reflex was also observed. After 300 mg./kg. the mice were in complete flaccid paralysis which began in the hind limbs. The righting reflex and muscular tone were completely absent. Corneal and pinna reflexes were decreased but not absent.

Other signs were first observed after 30 mg./kg. of L 1538: the respiration rate fell and slight cyanosis, lachrymation and hypothermia were observed. Depression of respiration increased progressively with dose and death was due to respiratory paralysis.

L 1460 produced substantially the same changes. Complete paralysis, however, was observed at a lower dose, namely, 200 mg./kg. Muscular tone was not completely lost and some rigidity, particularly of the forelimbs, was observed in some animals. In the first stage of paralysis some tremors also occurred. Side effects, such as depression of respiration, cyanosis, and mydriasis, appeared to be more marked than with L 1538.

L 1458 had about the same activity as L 1460, but no rigidity occurred and side effects were less marked.

No hyperexcitability before the loss of righting reflex and paralysis was ever seen in mice injected with any of the three compounds.

After oral administration the effects of L 1538, L 1460, or L 1458 were the same as after intraperitoneal administration, if the dose was increased by 75 to 100%.

The activity of the three substituted thiadiazoles was also compared with that of other paralysing drugs and benzimidazole. After intraperitoneal administration, 250 mg./kg. of mephenesin produced a complete relaxation of body muscles within 3 to 5 min. without tremor. The pinna reflex was lost, but autonomic effects were almost absent. Respiration was depressed during paralysis.

Meprobamate (300 mg./kg. intraperitoneally) caused paralysis after an induction period of 20 to 25 min. The duration of paralysis was about 2 hr. In most animals muscular tone did not completely disappear in the forelimbs and twitches were occasionally observed. The pinna reflex was retained.

Mice injected with 100 mg./kg. of benzimidazole intraperitoneally showed only slight muscular hypotonia and a slightly decreased corneal reflex. After 300 mg./kg. ataxia and an impairment of the righting reflex with a more consistent muscular hypotonia were observed.

Zoxazolamine (2-amino-5-chlorobenzoxazole) caused at a dose of 30 mg./kg. a very slight impairment of the righting reflex and at 100 mg./kg. a marked muscular hypotonia without signs of ataxia; in this respect it resembled mephenesin. The corneal and pinna reflexes were unaffected at this dose though higher doses abolished them.

The paralysing actions of L 1538, L 1460, and L 1458 in rats were qualitatively similar to those in mice, but were observed at lower doses. The mean paralysing dose and duration of action of L 1538, L 1460, L 1458, mephenesin, and meprobamate in mice and rats are given in Table I.

TABLE I

THE MEAN PARALYSING DOSES (PD50) AND DURATION OF ACTION OF L 1538, L 1460, L 1458, MEPHENESIN AND MEPROBAMATE ADMINISTERED INTRAPERITONEALLY TO MICE AND RATS

Comp.	Animals	PD50 (mg./kg.)	Approximate Duration (min.)
L 1538..	Mice	180	150
	Rats	150	120
L 1460..	Mice	140	>240
	Rats	70	120
L 1458..	Mice	130	90
	Rats	90	120
Mephenesin	Mice	180	12
	Rats	80	15
Meprobamate	Mice	200	90
	Rats	200	90

Effects on Cats and Dogs

When administered intraperitoneally to cats at 50 mg./kg., L 1538 caused slight ataxia and mydriasis lasting for about 90 min. After 100 mg./kg., a slight impairment of the righting reflex was observed as well as weakness of the hind limbs, salivation, emesis, and marked ataxia. L 1460 by the same route in the cat at 50 mg./kg. produced salivation, slight ataxia and mydriasis. After 100 mg./kg., the cat could not walk, ataxia was severe, and mydriasis occurred. The muscular tone was retained. After 150 mg./kg., the righting reflex was lost, voluntary movements disappeared and muscular tone decreased. Mydriasis was extreme and the pupillary reflex to light impaired. After 3 hr., isolated muscular twitches and tremors were observed. Respiration was depressed and death occurred within 8 to 10 hr. L 1458 induced less marked side effects. Salivation, mydriasis, and emesis were observed after 80 mg./kg. With 100 mg./kg., muscle relaxation was still moderate, but the righting reflex was markedly impaired and moderate ataxia was present. 150 mg./kg. caused weakness of fore and especially hind limbs, muscle relaxation preceded by rigidity, mydriasis, and salivation. Twenty hr. after the injection the animal appeared normal, and only spontaneous activity was still reduced. After oral administration the same effects were observed at about the same doses.

In the dog 25 mg./kg. of L 1460 intraperitoneally did not produce any change in behaviour. After 75 mg./kg., ataxia, vomiting and salivation were observed. After 100 mg./kg., the righting reflex was impaired and paralysis appeared within 90 min. Muscular tone was decreased although it was still present. After 150 mg./kg., respiration was affected and death occurred in extreme prostration within 10 hr.

L 1458, in doses of 75 mg./kg., caused ataxia, vomiting, salivation, and slight impairment of the righting reflex; after 125 mg./kg., motor incoordination was extreme, with weakness of limbs and moderate muscle relaxation. After 150 mg./kg., respiration was depressed but the animal survived. Twenty hr. after the administration, the movements of the dog were normal although signs of general depression were present.

Before the onset of neurotoxic signs no sedative effect was ever observed. Dogs injected with L 1460 and L 1458 tried to rise and walk although their movements were greatly impaired. Dogs treated with doses of meprobamate producing a similar degree of paralysis did not show any tendency to walk; and their curiosity and general reactivity to environmental changes was decreased. However, ataxia was present and sometimes emesis occurred.

Anticonvulsant Activities

Table II gives the mean doses of L 1538, L 1460, and L 1458 which prevented the appearance of the

TABLE II

ANTICONVULSANT ACTIVITY OF L 1538, L 1460, L 1458, MEPROBAMATE AND MEPHENESIN INJECTED INTRAPERITONEALLY INTO MICE. ALL DOSES ARE EXPRESSED IN MG./KG.

Comp.	Animals	ED50 (Electroshock)	ED50 Leptazol		ED50 Strychnine	
			Death	Convulsions	Death	Convulsions
L 1538 ..	Mice	70	>200	>200	<100	>200
	Rats	<12·5	>200	>200	~150	>200
L 1460 ..	Mice	20	>200	>200	85	130
	Rats	12	>100	>100	<100	>100
L 1458 ..	Mice	70	>200	>200	80	100
	Rats	18	>100	>100	<100	>100
Meprobamate	Mice	75	30	80	150	>500
	Rats	60	<100	>100	>100	>100
Mephenesin ..	Mice	85	>400	>400	350	400
	Rats	70				

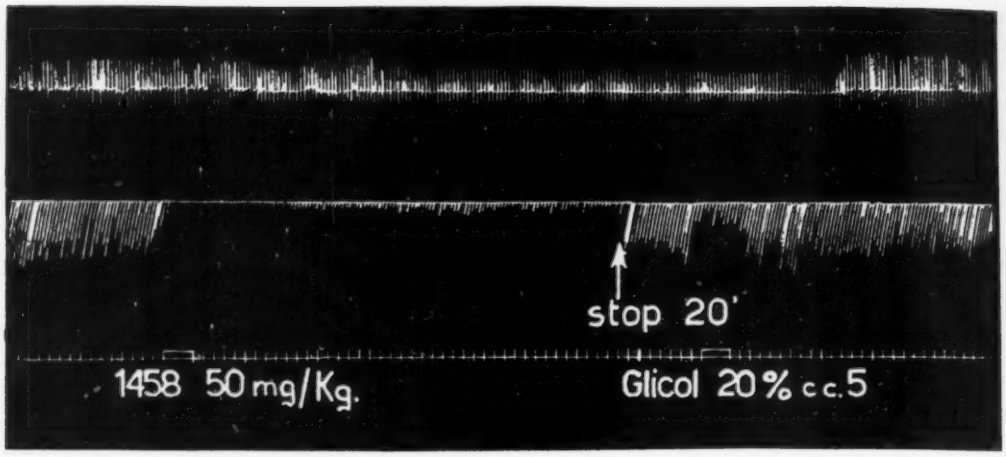

FIG. 1.—Cat, pentobarbitone anaesthesia. The effect of L 1458 (50 mg./kg. i.v.) on the knee jerk and flexor reflex. Upper trace, knee jerk; middle trace, flexor reflex; lower trace, time, 2 sec. and injection signal. Glicol 20% c.c.5 = control injection of 5 ml. of 20% propylene glycol. Stop 20' = drum stopped for 20 min.

tonic extensor component of maximal electrical seizures in mice and rats and the mean doses protecting both species from the convulsant and lethal effects of leptazol and strychnine.

The effectiveness of L 1458 in preventing the onset of the extensor phase of electroshock seizure was also confirmed in rabbits at a dose of 30 mg./kg. intraperitoneally.

Effect on Reflexes in Cats and Rabbits

The intravenous administration of L 1460 (20 mg./kg.) and L 1458 (20 mg./kg.) dissolved in 20% aqueous propylene glycol abolished the flexor reflex after a few minutes. The knee jerk was retained but usually reduced in amplitude (Fig. 1). The duration of action was about 35 min. These effects of L 1460 and L 1458 could also be observed after intraperitoneal injection of comparable doses.

Effect on Decerebrate Rigidity

The effects of L 1460 and L 1458 on decerebrate rigidity were tested at doses of 50 mg./kg. given intravenously in cats. Rigidity was usually reduced but occasionally it was abolished (Fig. 2). The duration of this action of L 1458 was substantially shorter than that of paralysis induced by the same dose in the intact cat. Rigidity was less affected by L 1460. Complete relaxation was observed after 50 mg./kg. of mephenesin or 100 mg./kg. of meprobamate.

Effect on Electroencephalogram

In preliminary experiments doses of 50 mg./kg. of L 1458 were injected intravenously or intraperitoneally in four cats. No consistent effects on electroencephalographic tracings were observed. Mephenesin (50 mg./kg.) induced slowing and increases in amplitude.

Potentiating Effect on Barbiturate-induced Hypnosis

Prolongation by L 1458 and L 1460 of hypnosis induced by pentobarbitone, thiopentone, quinalbarbitone, and hexobarbitone was studied in mice.

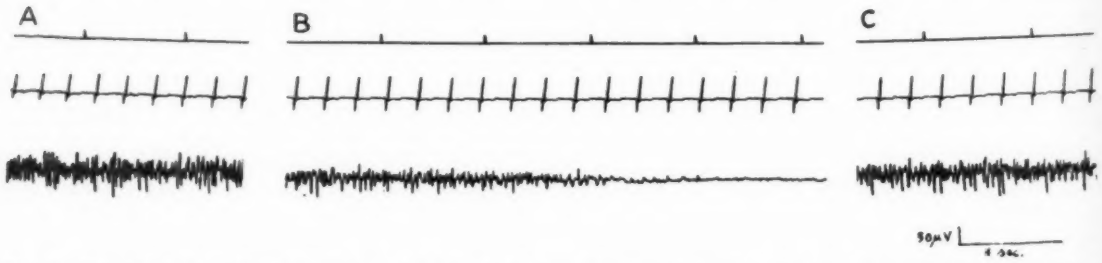

FIG. 2.—Decerebrate cat. Upper trace, time, 1 sec.; middle trace, electrocardiogram. Lower trace, electromyographic recordings from extensor muscles of the hind right leg. A, normal; B, at the end of an intravenous injection of L 1458 (15 mg./kg.); C, recording made 18 min. after the injection.

Table III gives the median sleeping time (ST50) as determined with various doses of thiadiazoles and meprobamate administered to groups of animals. L 1458 and L 1460 in doses of 15 mg./kg. significantly increased the sleeping time of mice treated with pentobarbitone and quinalbarbitone. In the same dose only L 1460 prolonged thiopentone and hexobarbitone hypnosis.

TABLE III

EFFECTS OF L 1458, L 1460 AND MEPROBAMATE ON THE SLEEPING TIME OF MICE INJECTED INTRAPERITONEALLY WITH SOME BARBITURATES

ST50=Median sleeping time (min.). C.L.=Confidence limits for 95% probability. R=Sleeping time ratio=$ST50_1/ST50_2$. fR=Ratio factor. The value of R will exceed the value of fR if the two median times being compared are significantly different for 95% probability (Litchfield, 1949).

Treatment	mg./kg.	No. of Animals	ST50 (C.L.)	R	fR
Pentobarbitone	40	40	34 (23–49)		
+L 1460 ..	15	40	215 (172–268)	6·32	1·53
+L 1458 ..	15	40	60 (49–73)	1·765	1·52
+Meprobamate	15	10	80 (70–91)	2·35	1·48
Hexobarbitone ..	80	10	35 (25–47)		
+L 1460 ..	15	10	125 (91–172)	3·57	1·56
+L 1458 ..	15	10	47 (26–84)	1·34	1·94
+Meprobamate	15	10	54 (51–57)	1·54	1·36
Quinalbarbitone	40	10	107 (74–155)		
+L 1460 ..	15	10	>310	—	—
+L 1458 ..	15	10	220 (157–308)	2·05	1·65
+Meprobamate	15	10	160 (140–183)	1·49	1·47
Thiopentone ..	45	40	18 (11–28)		
+L 1460 ..	15	10	100 (75–134)	5·52	1·71
+L 1458 ..	15	10	20 (11–36)	1·11	2·08
+Meprobamate	15	20	44 (23–82)	2·44	2·15

Under the same conditions meprobamate was less active than L 1460 and only slightly more active than L 1458. Neither the thiadiazoles nor meprobamate produced any impairment of motor activity in mice at the dose of 15 mg./kg. used in these experiments.

Antipyretic Activity

The antipyretic activity of L 1460 and L 1458 (20 and 40 mg./kg. intraperitoneally) was studied in 20 rabbits. Only L 1460 at 40 mg./kg. had an antipyretic action. The effect of the two doses of L 1458 and of the smaller dose of L 1460 was not significant. Under the same experimental conditions meprobamate, 20 and 40 mg./kg., also had a very poor effect.

Other Actions

In cats anaesthetized with pentobarbitone, L 1538 in a dose of 50 mg./kg. intraperitoneally produced within 15 min. a fall of blood pressure of about 10 mm. Hg, which lasted more than 30 min. The heart rate also fell. The response to carotid occlusion or to adrenaline or acetylcholine, intravenously, was not affected. The effect of 150 mg./kg. on blood pressure was greater and more prolonged. The response to vagal stimulation or carotid occlusion was slightly depressed. After 250 mg./kg. blood pressure, heart rate, and respiration were greatly depressed and death occurred within 60 to 90 min.

L 1458 had similar but larger effects on blood pressure and respiration. After 100 mg./kg., in two animals respiration was blocked and death would have occurred if artificial respiration had not been started. L 1460 was similar to L 1458.

The three compounds did not modify the response of the nictitating membrane to preganglionic cervical sympathetic stimulation.

On the smooth muscle of isolated rat ileum, L 1538, L 1460, and L 1458 showed a slight antagonism to acetylcholine at a bath concentration of 10^{-5} (w/v).

Toxicity

The acute toxicity of L 1538, L 1460, and L 1458 in mice was compared with that of mephenesin and meprobamate, after their administration intraperitoneally as suspensions in 5% gum acacia. Animals were observed for one week. The LD50 was calculated by the method of Litchfield and Wilcoxon (1949) and the values found are given in Table IV.

TABLE IV

ACUTE TOXICITY IN MICE BY INTRAPERITONEAL ADMINISTRATION

Confidence limits are given in parentheses (95% probability). All doses are expressed in mg./kg.

Comp.	LD50
L 1538	442 (507–387)
L 1460	264 (322–216)
L 1458	374 (408–343)
Meprobamate	719 (745–694)
Mephenesin	518 (559–480)

In a subacute toxicity test, two groups of three dogs each were given daily oral doses of 200 mg./kg. of L 1458 and 150 mg./kg. of L 1460 respectively for two weeks. Salivation, emesis, anorexia, ataxia, and variable degrees of paresis were seen after the first or the second administration and during all the period of treatment. At the end of the two weeks all the animals had lost weight and were in bad condition. There was prostration and paralysis. Urine analysis showed biliary pigments after the third day, and albumin near the end of the experiment. Complete blood counts during and at the conclusion of the experiments showed a progressive increase of neutrophils. The blood-clotting time was slightly increased after 8 days of treatment.

Gross and histological examination of the autopsied animals 15 days from the beginning of

treatment showed congestion of viscera and albuminous and fatty degenerative lesions of liver and kidneys.

A chronic toxicity test was carried out with three oral doses (30, 60, and 180 mg./kg.) of L 1458, administered daily to groups of ten rats each. 10% gum acacia was used to suspend the product; a volume of 2.0 ml./kg. was given. The duration of treatment was 17 weeks. During this period two animals receiving the dose of 180 mg./kg. died; no death was observed with the doses of 60 and 30 mg./kg. Only the rats given 180 mg./kg. of L 1458 grew significantly less well than controls: the mean body weight after 18 weeks of treatment was 280 g. and that of controls 330 g.

Blood counts during and at the end of the experiment did not differ significantly from observations before dosing.

Gross and microscopic examination of the autopsied animals at the end of treatment showed degenerative lesions in liver and kidney of some rats given 180 mg./kg.

Discussion

Studies of the action of the three thiadiazoles on the central nervous system have shown that these compounds, like other centrally-acting paralysing drugs, depress the activity of polysynaptic arcs of the spinal cord. Monosynaptic transmission is less affected. The action of L 1458 and L 1460 on spinal reflexes is similar to that of meprobamate, mephenesin, 2-aminobenzothiazoles, and other spinal depressant and centrally-acting paralysing drugs. However, hypnotics, anticonvulsants, analgesics, local anaesthetics, and drugs used in the treatment of paralysis agitans have been shown to depress polysynaptic reflexes without affecting the patellar reflex (Domino, 1956). In view of this, and of the presumed polysynaptic nature of most neuronal arcs concerned in central nervous activity, it may be concluded that selective action on spinal polysynaptic reflexes is almost aspecific. Such an action does not demonstrate an analogy between thiadiazoles and mephenesin-like compounds.

From gross observation in mice it also appears clearly that substantial differences do exist in the action of various central paralysing compounds. These differences concern principally their effects on motor functions and reflexes.

According to Goodsell, Toman, Everett, and Richards (1954) centrally acting relaxants of the mephenesin type abolish the pinna reflex earlier than the corneal reflex and show a smooth onset and a great deal of relaxation. Ataxia does not precede muscle weakness and paralysis. Our results with mephenesin are in full accord with these. Among the drugs studied by us only zoxazolamine resembled mephenesin. Meprobamate produced a good relaxation; however, in some animals it was less effective than mephenesin in this respect. Moreover meprobamate affected only slightly the pinna reflex. Berger (1954) reported a similar observation. Mice injected with benzimidazole show some ataxia before relaxation, and both the pinna and corneal reflexes are simultaneously and only moderately depressed.

L 1538, L 1460, and L 1458 produced slight relaxation, preceded by ataxia and impairment of the righting reflex. The corneal and pinna reflexes were simultaneously affected.

Other differences noted concerned the duration of paralysis which was about 10 min. with mephenesin, 2 hr. with meprobamate, 3 hr. with benzimidazole, about the same with L 1458 and L 1538, and more than 4 hr. with L 1460.

The different properties of centrally-acting muscle relaxants strongly suggest that such known and new compounds may act on different parts of the central nervous system and that they probably belong to different classes of drugs.

Other pharmacological studies on the three thiadiazoles L 1538, L 1460, and L 1458 confirm this point of view. The three products show anticonvulsant properties in mice and rats. They prevent electrical and strychnine-induced convulsions, but fail to protect animals against leptazol-induced seizures. In this respect thiadiazoles differ substantially from meprobamate which is especially effective in preventing leptazol-induced convulsions. On the contrary, they resemble mephenesin and benzimidazole which show a very poor anti-leptazol effect (Maffii *et al.*, 1958), and perhaps also the 2-aminobenzothiazoles, the anticonvulsant action of which against only electrically and strychnine-induced seizures has been reported (Domino, 1956).

L 1458 and L 1460 have shown some effect in reducing decerebrate rigidity in cats, but their effectiveness is poor compared with that of mephenesin. Moreover, L 1458 and L 1460 do not synchronize cortical electrical activity. From this point of view also their action differs from that of meprobamate (Hendley, Lynes, and Berger, 1954) and mephenesin (Toman and Davis, 1949) which slow and increase the amplitude of the cortical electroencephalogram. The 2-aminobenzothiazoles are also said to lack synchronizing action on the electroencephalogram (Funderburk,

King, Domino, and Unna, 1953; Funderburk, King, and Unna, 1953).

Thus, though the action of thiadiazoles on the spinal cord appears quite evident, the failure to antagonize the action of leptazol, the slight inhibition of decerebrate rigidity, the lack of electroencephalographic changes and the effects on behaviour demonstrate that any action of these drugs on higher levels of the central nervous system is doubtful.

On the other hand, highly effective centrally-acting muscle relaxants such as mephenesin, meprobamate, zoxazolamine and benzimidazole also displayed significant differences in activity on higher centres. The meaning of this fact is debatable and will remain so until knowledge of neuropharmacology is more adequate.

The similarity in activities of substituted thiadiazoles and benzothiazoles has been confirmed by our results. Common findings with the two series of products as described by Funderburk *et al.* (1953) or investigated by us are: spinal depressant activity, protection against electrical and strychnine-induced seizures and lack of synchronizing effect on the electroencephalogram. These findings give further support to the hypothesis (Maffii *et al.*, 1958) of pharmacological equivalence between the structures of aromatic nuclei condensed with heterocyclic rings, such as benzothiazoles, and the structures of heterocyclic nuclei containing aromatic substituents, such as the thiadiazole derivatives.

It is also interesting to observe that, in the thiadiazole series, substitution in position 5 either with phenyl or 2-thienyl leads to compounds that do not qualitatively differ in their pharmacological action.

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THE DISTRIBUTION OF METHYLPENTYNOL AND OF METHYLPENTYNOL CARBAMATE IN TISSUES AND BODY FLUIDS OF CATS

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A method is described for the estimation of methylpentynol and methylpentynol carbamate in body fluids and tissues. After intravenous administration, the distribution of these drugs throughout the body was found to be similar to that of ethanol. They entered cells, and crossed the blood-brain barrier and the placenta. They were excreted or secreted into the saliva, milk, gastric secretion, aqueous and vitreous humour, amniotic liquor, bile and urine. Methylpentynol was found in the expired air. Calculation of the volume of distribution and measurement of concentration in the tissues showed that both drugs were only slowly metabolized or excreted, suggesting the possibility that blood concentrations might rise cumulatively over a period of days.

Methylpentynol (3-methyl-1-pentyn-3-ol, Oblivon) is an unsaturated, tertiary alcohol introduced into therapeutics by Margolin, Perlman, Villani, and McGavack (1951), who also studied some aspects of its pharmacology as did Gialdroni and Grassi (1952). Perlman and Johnson (1952) and Perlman, Sutter, and Johnson (1953) investigated the metabolism, distribution, and excretion of methylpentynol. They suggested that the drug was eliminated rapidly from the blood and not stored in the tissues, implying rapid metabolism. For instance, they found that, if dogs were given methylpentynol (200 mg./kg. intravenously), only 20% of the total dose could be recovered from venous blood 10 min. later and 12% after 25 min.; after 2 hr. none could be detected. In rats given methylpentynol (800 mg./kg. orally) and killed at the time of maximum sedation, less than 7% of the total dose could be recovered from the tissues. When the sedative effect had worn off, no methylpentynol could be recovered from the tissues. Moreover, although methylpentynol is volatile, no trace could be detected in the expired air collected for 7 hr. after the administration of 200 mg./kg. of the drug orally to a dog. It has therefore been contended that, though methylpentynol is an alcohol, the body treats it differently from the lower alcohols such as ethanol; its metabolism is considerably more rapid and it is not stored in the tissues. In contradistinction are the clinical accounts of the use of methylpentynol (May and Ebaugh, 1953; Marley and Chambers, 1956) and the more recent derivative methylpentynol carbamate (Bartholomew, Chappell, Marley and Chambers, 1958) from which it would appear that an accumulation of both these substances may occur in humans, leading to toxic phenomena.

Because of the discrepancy between the animal results and those seen clinically in man, we have re-examined the distribution and excretion of methylpentynol in animals. Although the results of experiments on the metabolism and fate of methylpentynol carbamate have not been published, its activity is said to depend on the conversion of the substance to methylpentynol in the body. We have therefore also studied the distribution of methylpentynol carbamate in animals.

METHODS

Reagents

All reagents used were of Analar standard.

Alkaline Silver Reagent.—250 ml. 0.1 M-$AgNO_3$ and 30 ml. 6 N-NaOH were mixed in a 500 ml. volumetric flask. Sufficient concentrated NH_4OH was added to dissolve the precipitate and a further 20 ml. was added in excess. The solution was made up to 500 ml. with distilled water and stored in a dark bottle. Alkaline silver reagent (0.025 M) was prepared by adding an equal volume of distilled water to this solution.

Potassium Thiocyanate.—KCNS (10 g.) was dissolved in water and made up to 1 l., to give an

approximately 0.1 M solution which could then be diluted to 0.05 or 0.025 M as required.

Ferric Ammonium Alum Indicator.—To a cold saturated solution of ferric alum enough nitric acid was added for the brown colour to disappear (approximately 3 g. of the alum, 10 ml. of water, and 2 ml. of 6 N-HNO_3). The same amount of indicator was used for all titrations, namely 0.2 ml.

Estimation of Methylpentynol

The method used for estimating methylpentynol was based on that of Perlman and Johnson (1952) as modified by Perlman *et al.* (1953). They distilled the methylpentynol from a sample into the alkaline silver reagent. The acetylene group of the methylpentynol reacted quantitatively with the alkaline silver reagent to form a precipitate. This precipitate was collected, washed, redissolved in nitric acid, and its silver content determined colorimetrically using Rhodamine reagent after a 500-fold dilution. In our hands this method proved unreliable, recoveries being very widely scattered (50 to 250%). We therefore developed the following technique. The minced tissue or fluid sample was placed in a boiling tube (" sample tube " in Fig. 1) and the volume made up to approximately 15 ml. with distilled water. A trace of silicone antifoam agent was added. The sample tube was heated in the water bath at a temperature of 80 to 85° and a stream of air bubbled through. This

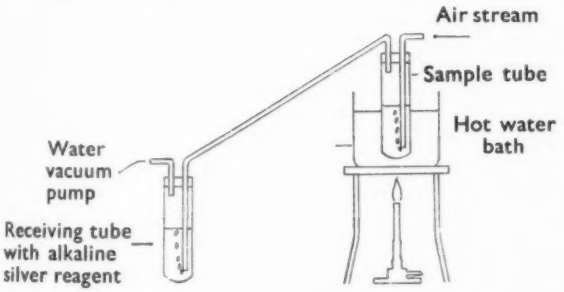

FIG. 1.—Apparatus for distillation of methylpentynol and methylpentynol carbamate from minced tissue samples or body fluids.

volatilized the methylpentynol which was carried in the airstream into 5 ml. of the alkaline silver reagent diluted with 10 ml. of distilled water, where it precipitated with the silver as in the method of Perlman *et al.* (1953). In preliminary experiments, the sample tubes were heated for 30 min., 60 min., and 2 hr. It was found that all the methylpentynol distilled over within 30 min., so this period was used. Six samples were treated at the same time, the air being drawn through both the sample and the receiving tube by suction from a water vacuum pump attached to a manifold. The alkaline silver reagent was then centrifuged at 3,000 rev./min. for 15 min., in order to pack the precipitate at the bottom of the tube. The supernatant layer was carefully decanted, the precipitate washed, and the combined supernatants acidified with 2 ml. concentrated HNO_3. Iron alum indicator (0.2 ml.) was added and the silver estimated by titration with KCNS. A standard curve was obtained by adding 0.25, 0.5, 1.0, 2.0, 4.0 mg. of methylpentynol respectively to 5 ml. of the silver reagent, centrifuging and then back titrating the excess silver present in the decanted supernatant. The amount of thiocyanate required was plotted against the amount of added methylpentynol (Fig. 2). This curve was used for

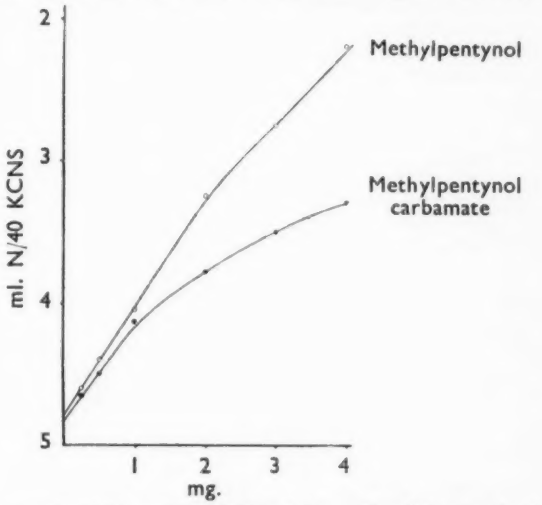

FIG. 2.—Standard curves for 0·25 to 4·0 mg. of methylpentynol and methylpentynol carbamate added directly to alkaline silver reagent (see text).

estimating the amount of methylpentynol present in the samples of body fluids or tissues. New recovery curves were plotted each time fresh solutions were prepared. In this way the need for an exact standardization of a silver or thiocyanate solution was eliminated.

Estimation of Methylpentynol Carbamate

Hydrolysis with 0.2 N-H_2SO_4 was essential for the liberation of methylpentynol from the carbamate. This acid was chosen because of its low volatility. The procedure was identical with that for methylpentynol, except that before the distillation procedure began 2.0 to 3.0 ml. of 3 N-H_2SO_4 was added to the sample tube. Standard curves were obtained for the same amounts of added drug as with methylpentynol (Fig. 2). Alkaline hydrolysis of the carbamate provided better recoveries than acid hydrolysis from distilled water but very variable recoveries from blood.

Recovery from Tissue Samples

Control tissue samples usually of 5 to 20 g. from cats anaesthetized with chloralose or pentobarbitone sodium were minced and extracted by the distillation procedure. No precipitation of the alkaline silver reagent occurred except with distillates of minced foetal tissue samples of about 70 g. These gave a small amount of black precipitate and at the same time a sulphurous smell could be detected in the stream of air from the sample tube. To minimize

liberation of gaseous sulphides, lead nitrate was added to the large foetal tissue samples. Propylene glycol was also added to blank tissue samples before distillation, since it was sometimes used as an injection vehicle for the carbamate. No precipitation of the silver reagent occurred. Having shown that tissue blank values were satisfactory, the next step was to estimate the recovery of known amounts of methylpentynol and carbamate. Between 0.25 and 2 mg. of methylpentynol or the carbamate were added to 10 ml. water, 10 ml. stored cat blood or minced cat tissue (liver, thigh muscle, diaphragm, lung, kidney, brain, and heart: the samples varied between 8 and 17 g.). The samples were then extracted by the distillation procedure. The mean recoveries and standard deviations are shown in Table I. It will be noted that the mean recovery for methylpentynol from blood over a range of 0.25 to 2 mg. of the drug was 98%. This compares favourably with a mean recovery of 73% of added material found by Perlman and Johnson (1952) using their method. The 65% recovery of methylpentynol carbamate was disappointing, and the reason for this has not yet been found. Concentrations of drugs in tissues quoted below have not been corrected for this low recovery of methylpentynol carbamate, and are therefore minimum values.

Recovery from Fresh Blood Samples

To determine whether either methylpentynol or the carbamate was metabolized in fresh blood, samples of blood were taken from an anaesthetized cat and methylpentynol or carbamate was added. After incubation for periods of up to 1 hr. in capped centrifuge tubes at 37° and centrifugation for 15 min. the plasma and red cells were analysed. There was no loss of either methylpentynol or methylpentynol carbamate from blood under these conditions. It was therefore presumed that drug concentrations in blood samples did not change after the samples were taken.

Perlman *et al.* (1953) suggested that the ammoniacal silver reagent may lose ammonia during the estimation and allow precipitation of silver carbonate. To counteract this they sometimes added 2 ml. of concentrated NH_4OH to the reagent during the distillation. We repeated this procedure with solutions which were bubbled for 30 min., and also by adding NH_4OH to the sample tube to charge the airstream with ammonia vapour. In both instances the addition of ammonia reduced the recovery of methylpentynol to between 0 and 20%. Thus the addition of more ammonia to the silver reagent changes its properties and invalidates the method. To test this further the methylpentynol and the carbamate were added directly to the silver reagent together with 1 ml. of concentrated NH_4OH. The reaction of the methylpentynol with the silver reagent was inhibited so much that only about 30% of the expected precipitate was formed. It was also possible to redissolve the precipitate once it had formed by adding more NH_4OH. Tissue and blood blank experiments also showed that the addition of excess NH_4OH was unnecessary, for had any silver carbonate been precipitated it would have shown as a positive reaction.

As a further test of the specificity of the method, a collection of 80 specimens obtained from human tissues was analysed for methylpentynol or methylpentynol carbamate. At the time of analysis it was not known which, if any, of these samples contained methylpentynol or the carbamate. Of the 80 samples 15 were correctly found to contain methylpentynol or its carbamate, one gave a false positive and the other 64 were correctly found to contain no drug.

Animal Experiments

The cats used were anaesthetized either with chloralose (80 mg./kg.) after ether induction, or with intraperitoneal pentobarbitone sodium (30 mg./kg.). In two or three instances infusion of methylpentynol or methylpentynol carbamate was started during ether anaesthesia and anaesthesia then maintained by this infusion. Spinal cats prepared by the method of Kosterlitz, Krayer, and Mattalana (1955) were used in some experiments. Rectal temperature was maintained at 38°. Methylpentynol or the carbamate was given either by infusion at the rate specified or as a single intravenous injection. Arterial blood samples were taken from a polythene cannula in the femoral artery. Tissue samples were obtained by dissection of the specimen between the ligatures. In the pregnant animals 3 to 5 foetuses were usually present. These were removed one by one to coincide with the withdrawal of the tissue or fluid samples. A uterine horn was opened, the amnion incised, the contained

TABLE I

RECOVERY OF METHYLPENTYNOL OR METHYLPENTYNOL CARBAMATE FROM DISTILLED WATER, CAT WHOLE BLOOD AND CAT TISSUES

The values are expressed as % ± s.d. The number of estimations made are given in brackets.

	Methylpentynol				Methylpentynol Carbamate			
Amount added (mg.) ..	0·25	0·5	1·0	2·0	0·25	0·5	1·0	2·0
Recovered from:								
Distilled water	90±0·0 (4)	96±4·0 (3)	90±0·0 (3)	93±5·3 (3)	68±9·3 (3)	60±8·3 (5)	50±0·0 (3)	60±7·1 (3)
Cat whole blood ..	89±7·7 (5)	100±2·0 (5)	102±3·4 (5)	102±1·6 (3)	68±9·2 (6)	64±12·0 (5)	66±10·4 (3)	60±0·0 (3)
Cat tissues	105±8·6 (4)	97±4·3 (4)	98±8·9 (4)	97±8·7 (4)	57±5·9 (3)	58± 6·5 (3)	75±15·2 (5)	76±18·0 (5)

fluid collected, and the foetus and placenta abstracted. The horn was then closed with a tissue clamp, as was the abdomen, until the next specimen was required. In four experiments, the expired air was collected through a respiratory valve in large polythene bags. The contents of the bag were then pumped slowly through the alkaline silver reagent in order to measure the methylpentynol content. The same procedure applied to the contents of a bag filled with London air caused no precipitation of the silver reagent.

Treatment of Samples

Blood samples of 4 to 5 ml. were collected in glassware after intravenous injection of heparin (10 mg./kg.). The final blood sample was obtained by maximal exsanguination. Because methylpentynol is volatile, all the blood samples were kept in closed tubes from the time of withdrawal to the time of estimation. When both blood and plasma values were required, 10 ml. samples were collected and 4 ml. of the blood was immediately lysed with water and used later for whole blood estimation. The rest was centrifuged at 3,000 rev./min. for 15 min., in order to obtain plasma and cell samples. Both whole blood and plasma samples were stored in the deep freeze. Tissue samples were minced with scissors and stored in sealed bottles in the deep freeze until the estimation was performed. They were then warmed to room-temperature, mixed with water and transferred to the boiling tube (or to a conical flask if the sample was large).

The sensitivity of the method depended upon the total quantity of fluid or tissues taken for estimation. Thus, the method can detect as little as 0.1 mg. methylpentynol and this could be in a small amount of tissue, in which instance the sensitivity would be low, or in, say, 20 g. of tissue, allowing the detection of 0.005 mg./g. For the work described in this paper, concentrations of both methylpentynol and the carbamate were measured in samples usually greater than 4 ml. fluid or 5 g. tissue, allowing the detection of 0.02 mg./g. The accuracy of the method depends upon titration technique and the recovery figures. The excess silver was titrated to the nearest drop (0.03 ml.) of thiocyanate, equivalent in the lower range of the titration curve to 0.03 mg. methylpentynol. For samples of 5 g., this means that the accuracy of the method is within 0.01 mg. methylpentynol. The recovery figures for the carbamate were less satisfactory, and in consequence the accuracy is more difficult to assess. Because of this, the concentrations of the carbamate have been given to the nearest 0.05 mg./g. or ml. The concentrations of methylpentynol have been expressed to the nearest 0.01 mg./ml.

For injection and infusion, pure methylpentynol was dissolved in 0.9% saline (w/v). The methylpentynol carbamate was also dissolved in saline for infusion experiments, but for single injections it had to be dissolved in 60% (v/v) aqueous propylene glycol in order to reduce the volume injected.

Results

Fluid and tissue concentrations of both drugs were measured in cats following intravenous injection (up to 200 mg./kg.) and slow intravenous infusion (up to 350 mg./kg.). The blood concentrations are given in Table II. In Experiment

TABLE II

BLOOD CONCENTRATIONS OF METHYLPENTYNOL AND METHYLPENTYNOL CARBAMATE IN THE CAT IN RELATION TO TIME OF SAMPLING AFTER ADMINISTRATION

All cats used were females except those in Expt. Nos. 2, 5, and 7, which were male. Values obtained from exsanguination samples (Expt. Nos. 5, 6, 8, 9, 10 and 13) are given in bold type. The concentrations of methylpentynol or methylpentynol carbamate in the blood at time of sampling are expressed as mg./ml. of whole blood.

Expt. No.	Preparation	Weight (kg.)	Dose Administered (mg./g.)	Blood Concentrations at Time (in min.) After End of Drug Administration								Other Final Samples
				1	2	5	10	20	40	60	100	
Methylpentynol												
1	Chloralose (80 mg./kg.)	2·8	0·1	—	0·35	—	0·15	0·13	0·10	—	—	0·11 (160 min.)
2	,, ,,	4·8	0·1	0·66	0·40	0·34	0·18	0·16	0·09	—	—	—
17	,, ,,	3·8	0·2	0·56	0·49	0·34	0·26	0·20	0·19	—	—	—
3	,, ,,	3·0	0·25	—	0·74	0·49	0·40	0·33	—	—	—	—
6	,, ,,	2·6	0·35 in 45 min.	0·58	—	—	0·36	0·32	0·32	**0·36**	—	—
8	,, ,,	4·1	0·32 in 20 min.	0·25	—	—	0·17	—	0·15	**0·20**	—	—
9	Methylpentynol + 20 mg./kg. chloralose	2·5	0·3 in 15 min.	—	—	0·35	—	—	0·24	—	0·23	0·19 (160 min.) **0·27 (180 min.)**
10	,, ,,	3·0	0·2	—	—	—	—	—	—	—	—	0·29 (240 min.) **0·30 (280 min.)**
5	Spinal	3·4	0·2	—	1·45	0·88	0·41	0·28	0·19	0·15	—	**0·18 (120 min.)**
7	,,	3·1	0·2	—	—	—	—	0·14	—	—	—	0·05 (120 min.)
4	Pentobarbitone sodium (60 mg./kg.)	2·3	0·2	—	0·82	0·60	0·47	0·29	—	—	—	—
Methylpentynol carbamate												
12	Chloralose (80 mg./kg.)	2·6	0·2	—	—	—	0·45	—	0·15	—	0·10	—
13	,, ,,	3·1	0·2	—	—	—	0·35	—	0·10	—	—	0·05 (120 min.) **0·10 (180 min.)**
14	,, ,,	2·6	0·35 in 90 min.	—	0·20	—	—	—	—	0·15	—	—
18	Methylpentynol carbamate	4·1	0·2	—	—	—	—	0·25	—	0·15	—	—
11	Spinal	2·0	0·2	1·30	0·25	0·20	0·20	0·15	0·15	—	—	—

Nos. 1, 2, and 11, both plasma and blood concentrations were measured. They were found to be equal within 2 min. for methylpentynol and within 10 min. for the carbamate. For the other experiments the concentration in whole blood was assumed to be equal to the plasma concentration. The blood concentrations of both methylpentynol and the carbamate declined rapidly within the first 10 min., and then more slowly (Table II and Fig. 3).

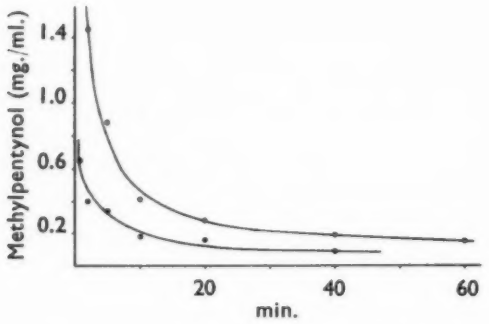

FIG. 3.—Blood concentrations of methylpentynol after single intravenous injections of 200 mg./kg. (O, Expt. No. 5); 100 mg./kg. (●, Expt. No. 2) plotted against time in min. after injection.

Table III shows the volumes of distribution at various times and it will be seen that methylpentynol was distributed throughout the total extracellular fluids within 2 min. of injection and had entered the cells within 10 min. At 40 min., the volume of distribution was about 100%, suggesting that the drug was either being concentrated by cells or being metabolized or excreted. The values for the carbamate show a similar rate of diffusion throughout the body compartments.

TABLE III

VOLUME OF DISTRIBUTION

The values represent the % of body weight through which the drug is distributed as calculated from blood concentrations at specified intervals after injection. The carbamate results are calculated on the assumption that 65% of the actual drug concentrations were measured.

Expt. No.	Time (min.)				
	1	2	10	20	40
Methylpentynol					
1	—	29	67	77	100
2	15	25	56	63	110
3	—	34	63	76	—
4	—	25	43	69	—
5	—	14	49	74	105
7	—	—	—	140	—
Methylpentynol carbamate					
11	10	38	65	86	—
12	—	—	30	—	86
13	—	—	40	—	130

TABLE IV

CONCENTRATION IN TISSUES AND BODY FLUIDS OF CATS AT VARIOUS TIMES AFTER SINGLE INJECTIONS OR INFUSIONS OF METHYLPENTYNOL AND METHYLPENTYNOL CARBAMATE

Single injections were given in Expt. Nos. 7, 12, 13, 17 and 18, and infusions in Expt. Nos. 6, 8, 9, 10, 14, 15 and 16. Concentrations and doses are given as mg./g. or mg./ml., except with gastric contents where the weight (in mg.) of the drug in lumen of stomach at end of the experiment is recorded and with expired air where the value denotes the weight (in mg.) of the drug collected in first hour. In Expt. No. 6, 60 min. after infusion of 0·35 mg./g. of methylpentynol, the concentration in blood was 0·32 mg./ml., and in milk 0·40 mg./ml. In Expt. No. 14, 2 min. after infusion of 0·35 mg./g. of methylpentynol carbamate, the concentration in blood was 0·20 mg./ml. and in milk 0·20 mg./ml. In Expt. No. 15, 2 min. after infusion of 0·07 mg./g. of methylpentynol, the concentration in blood was 0·15 mg./ml. and in saliva 0·16 mg./ml. In Expt. No. 16, 90 min. after infusion of 0·25 mg./g. of methylpentynol, the concentration in the gastric secretion was 0·50 mg./ml.

	Methylpentynol					Methylpentynol Carbamate		
Expt. No. ..	7	8	9	10	17	12	13	18
Total dose (mg./g.)	0·2	0·18	0·32	0·30	0·20	0·20	0·20	0·20
Time after administration (min.)	120	2	180	280	10	120	180	50
Tissue								
Final blood concentration ..	0·05	0·15	0·19	0·29	0·20	0·10	0·05	0·15
Brain	—	—	0·35	0·08	—	0·30	0·05	—
Heart	—	—	—	0·28	—	0·15	0·20	—
Diaphragm ..	0·14	—	0·24	0·26	—	0·05	0·15	—
Thigh muscle ..	—	—	—	0·12	—	0·10	0·05	—
Mesenteric fat ..	0·19	—	0·45	0·22	—	0·10	0·10	—
Lung	0·19	—	0·30	0·15	—	0·25	0·15	—
Liver	0·20	—	—	0·17	—	0·10	0·25	—
Kidney	0·16	—	0·33	0·13	—	0·25	0·20	—
Spleen	0·08	—	—	0·17	—	—	—	—
Intestine	0·17	—	—	—	—	—	—	—
Skin	—	—	—	0·10	—	—	0·05	—
Bone	—	—	—	0·06	—	—	0·05	—
Foetus	—	—	—	0·18	—	0·15	—	—
Placenta	—	—	—	0·06	—	0·15	—	—
Bile	0·0	—	0·0	Trace	—	0 0	—	0·15
Urine	0·16	—	—	0·33	—	—	0·10	—
Aqueous humour	—	—	0·10	—	0·19	0·0	0·0	0·10
Vitreous humour	—	—	—	—	0·15	0·10	0·10	—
Amniotic liquor	—	0·14	—	0·29	—	0·10	—	—
Gastric contents	0·8	—	4·5	—	—	—	5·0	—
Expired air ..	2·5	0 85	—	—	—	0·0	0·0	—

To determine whether the methylpentynol and carbamate were being metabolized, concentrated, or excreted, fluid and tissue concentrations were measured and the results are shown in Table IV. Both substances were found in saliva, gastric secretion, milk, aqueous and vitreous humour, amniotic liquor, bile, and urine. Entry into the lumen of the stomach took place during secretion of acid gastric juice (induced by histamine infusion) and also when the stomach was not secreting. The intestinal contents, after ligation of the pylorus, were also assayed for methylpentynol and the carbamate, but only traces were found.

The high concentration of methylpentynol in the milk (0.4 mg./ml.) at the end of Experiment No. 6 suggested that the drug diffused into preformed milk. This was confirmed in Experiment No. 14 where immediately after ending the infusion of the drug 2 mg. methylpentynol carbamate

was found in 10 ml. (0.2 mg./ml.), a volume of milk which must have been largely preformed.

The tissue concentrations shown in Table IV indicate that both drugs reach all tissues examined in comparable amounts. As these were obtained from 2 to 4 hr. after injection of the drug when, in some experiments at least, the blood concentrations had declined, it is probable that the methylpentynol and carbamate were not being metabolized to any great extent. For instance, in Experiment No. 7 the blood concentration was 0.05 mg./ml., but the concentrations of methylpentynol in the tissues varied between 0.08 and 0.20 mg./ml., the highest being in the liver, fat, and lungs. If the average tissue concentration in this cat was 0.15 to 0.20 mg./g., then of the 200 mg./kg. injected 2 hr. beforehand 150 to 200 mg./kg. (75 to 100%) was still left. The results of the other experiments also indicate that 70 to 100% of the initial dose remained in the tissues 200 to 300 min. later; similar results were obtained with the carbamate.

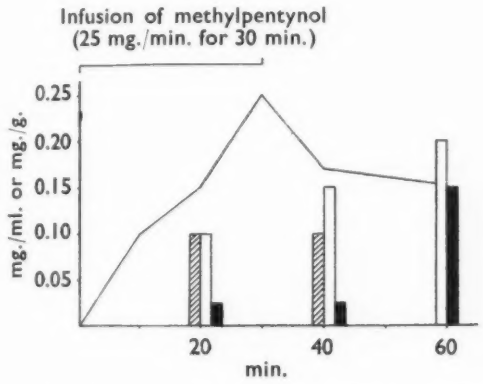

FIG. 4.—Expt. No. 8. Concentrations of methylpentynol in body fluids or tissue plotted against time following infusion of the drug. The line represents the concentration (in mg./ml.) in blood; the solid columns, the concentration (in mg./ml.) in amniotic liquor; the open columns, the concentration (in mg./g.) in the foetus; the crosshatched columns, the concentration (in mg./g.) in placental tissue.

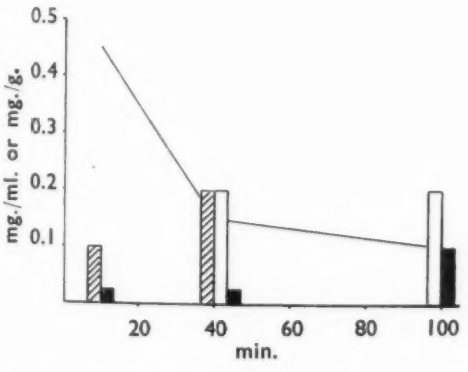

FIG. 5.—Expt. No. 12. Concentrations of methylpentynol carbamate plotted against time following a single intravenous injection of the drug. For explanation of the line and columns see Fig. 4.

Figs. 4 and 5 show that both methylpentynol and methylpentynol carbamate have general access not only to all body tissues examined but also to foetal tissues and eventually to amniotic fluid. Although most foetal tissues did not provide large enough samples to detect either drug, foetal liver and foetal heart were found to contain about the same concentrations of both drugs as the whole foetus. Thus we can say that there is no barrier to the general distribution of both drugs throughout the body.

In two experiments the expired air from the animal was collected in polythene bags and was later pumped through the alkaline silver reagent. Up to 0.4% of the total dose of methylpentynol was excreted into the expired air within 1 hr. of intravenous administration. In two similar experiments following the injection of methylpentynol carbamate, no methylpentynol was recovered from the expired air. Furthermore, although we always looked for methylpentynol in fluid and tissue samples after the administration of carbamate, none was detected, except once when a trace was found in a kidney tissue sample.

All these results indicated that neither methylpentynol nor the carbamate was rapidly metabolized.

DISCUSSION

The method described for estimating methylpentynol and the carbamate was based on that described by Perlman *et al.* (1953). The titration of silver left uncombined with methylpentynol gave more consistent results than colorimetric estimation of the amount of silver precipitated. The other advantage of this modified method was that as little as 0.1 mg. of methylpentynol could be estimated, a tenfold increase of sensitivity over the method of Perlman and Johnson (1952). The addition of further ammonia to the silver reagent during the distillation, a procedure recommended in the original method, was omitted when it was found to inhibit the formation of the precipitate and make the method completely unreliable. This may in part explain why Perlman *et al.* (1953) were unable to detect methylpentynol in the expired air of the dog.

There was no difference between the plasma concentration and the whole blood concentrations

of methylpentynol and the carbamate within 10 min. of intravenous injection. This does not necessarily mean that the alcohol and the carbamate were evenly distributed between blood cells and plasma within 10 min., for further equilibration may have taken place during the 15 min. centrifugation. If this time is taken into account the results show that both drugs were evenly distributed throughout blood cells and plasma within 25 min. of injection and possibly before this.

The main conclusions that can be drawn are first that both methylpentynol and methylpentynol carbamate have free access to all compartments of the body including the uterine contents, and second that excretion or inactivation of the substance in cats is slow. That general anaesthesia does not depress the metabolism or excretion of methylpentynol can be seen from Table II, for although the blood levels at various times were similar in the different cats, some were either spinal or were anaesthetized only with methylpentynol and some had chloralose or pentobarbitone sodium. These conclusions differ from those of Perlman *et al.* (1953), who used rats and dogs.

The general distribution of methylpentynol and its carbamate closely followed that of the lower alcohols. They readily crossed the placenta, both being recovered from the foetus, the amniotic liquor and the placenta within 10 to 20 min. of intravenous injection. The concentration in the amniotic liquor was initially lower than in the foetus but gradually increased as the experiment progressed. Nicloux (1899) demonstrated that alcohol passed from the mother to the foetus and that concentrations in the maternal and foetal blood were of the same order. Olow (1922) found appreciable quantities of alcohol in human foetal blood within 12 min. of injection; maternal and foetal blood reached similar concentrations 40 min. after ingestion, the subsequent rate of disappearance of both being identical. Although we were unable to procure sufficient cerebrospinal fluid to find either the speed of entry or the concentration of the drugs at this site, we did find concentrations in the brain similar to those in other tissues. The fact that both drugs were recoverable from the aqueous humour implies that they had entered the cerebrospinal fluid. Davson (1957) calls attention to the similarity between the blood/aqueous barrier and the blood/cerebrospinal fluid barrier. Even the passage of both drugs into the stomach lumen following intravenous injection is similar to that of ethanol; Grehant (1903) demonstrated alcohol excretion into the stomach lumen after intravenous injection as did Lukas (1930) following rectal administration. The fact that methylpentynol and the carbamate cross the stomach wall but not the duodenal wall implies that absorption of the drug takes place from the stomach following oral administration, as does the absorption of ethanol.

Ethanol is excreted in the expired air and we have found that methylpentynol is also excreted in the breath in amounts up to 0.4% of the total dose within 1 hr. of administration.

The volume of distribution of both drugs after single intravenous injections indicates that they are rapidly distributed throughout the total extracellular fluids and enter cells within 20 min. This is also shown by the tissue concentrations following the administration of both drugs. The fact that the tissue concentrations of both methylpentynol and the carbamate are sometimes higher than the blood concentrations after a period of 60 to 120 min. indicates that, after entry into the cells, there is more difficulty in getting out again. This difficulty seems to be overcome if too much blood is taken from the animal, for terminal values obtained by gross exsanguination are always higher than the penultimate values. This is reminiscent of the behaviour of potassium which is normally concentrated to a high degree by the cells. Anoxia, severe haemorrhage or other traumatic procedures lead to leakage of potassium from the cells into the blood (Scudder, Smith and Drew, 1939; Winkler and Hoff, 1943).

From these results in the cat it might be expected that methylpentynol and the carbamate would accumulate if given over a long period of time to man, and Bartholomew, Bourne, and Marley (1958) have shown that daily doses of methylpentynol and carbamate lead to rising blood concentrations in man.

Except for traces in the kidney, methylpentynol was never found in blood following administration of the carbamate, nor was there any respiratory excretion of the alcohol. This seems to be good evidence that the carbamate is acting as such and not through breakdown into methylpentynol as has been suggested. Thus the relationship between methylpentynol and methylpentynol carbamate seems to be similar to that between ethyl alcohol and ethyl carbamate (urethane). Furthermore, the general distribution of methylpentynol and its carbamate are similar to that of ethyl alcohol.

We should like to thank Professor W. D. M. Paton, F.R.S., for his stimulating interest throughout this work, also British Schering Ltd. for a generous supply

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CARDIAC OUTPUT DURING EXCITATION OF CHEMOREFLEXES IN THE CAT

BY

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In cats under chloralose anaesthesia the reflex fall of blood pressure and heart rate caused by injection of veratrine, amidines, diphenhydramine, or ethyl acetoacetate was accompanied by a fall in cardiac output. After veratrine and amidines there was a fall in mean pulmonary arterial pressure and after veratrine no significant change in pulmonary vascular resistance. After diphenhydramine and ethyl acetoacetate there was a rise in mean pulmonary arterial pressure and after diphenhydramine an increase in pulmonary vascular resistance. The effects of veratrine and amidines, but not those of diphenhydramine and ethyl acetoacetate, were abolished by section of the vagi. The main change leading to the fall of cardiac output after amidines was bradycardia.

The chemoreflexes which can be elicited from receptors in the heart and lungs (Dawes and Comroe, 1954) are manifested by a fall in heart rate, a fall in systemic arterial pressure, rapid shallow breathing or arrest of respiration and, frequently, increased bronchial tone. It was not known if they had any effect on the pulmonary circulation or cardiac output. It was therefore decided to make direct measurements of pulmonary arterial blood flow and pressure during the administration of drugs which stimulate these receptors.

METHODS

Cats were anaesthetized with chloralose (50 or 60 mg./kg. i.v.). The chest was opened widely through a midline incision and positive pressure ventilation was maintained with a Starling Ideal pump. Heparin (10 mg./kg., Boots) was given and either a bubble flowmeter or a density flowmeter (Dawes, Mott, and Vane, 1953) was inserted into the main or left pulmonary artery or left lower lobe artery. Since the results obtained with the two types of flowmeter were identical it has not been stated in the tables which type was used for a given experiment. Blood flow in the main pulmonary artery is equal to the total cardiac output and a mean value in these experiments was 280 ± 68.7 ml./min. or 114 ± 20.6 ml./min./kg. (16 cats). The bubble flowmeter consisted of a loop of polyethylene tubing marked in ml. with a bubble trap at the distal end. The pressure drop across the flowmeters was as follows. The drop with the bubble flowmeter for main pulmonary artery was 6 mm. Hg at a flow rate of 800 ml./min. (saline) and for the left pulmonary artery was 4 mm. Hg at a flow rate of 150 ml./min. (saline). The drop with the density flowmeter for main pulmonary artery was 4.4 mm. Hg at a flow rate of 1,840 ml./min. (saline) and for the left pulmonary artery was 2 mm. Hg at a flow rate of 128 ml./min. (saline). The details of insertion of these flowmeters have already been described (Barer and Nüsser, 1957). Pulmonary arterial pressure was recorded with a mercury manometer attached to the flowmeter circuit close to the distal cannula. Left atrial pressure was read directly in cm. of blood in a vertical tube tied into the auricular appendage. Pulmonary vascular resistance was calculated in Peripheral Resistance Units from the formula: Resistance (PRU) = Mean pulmonary arterial pressure − mean left atrial pressure (mm. Hg)/pulmonary blood flow ml./min.

In six experiments a Starling resistance was inserted into the main pulmonary artery and the lungs were perfused at constant pressure (Barer and Kottegoda, 1958).

We are indebted to Dr. F. L. Rose for phenyldiguanide hydrochloride, to Professor O. Krayer for veratridine and to Dr. W. F. Short for 2-α-naphthylethyl*iso*thiourea hydrochloride. Diphenhydramine hydrochloride (Parke, Davis), ethyl acetoacetate (B.D.H.), Chlorbismol, a fine suspension of bismuth oxychloride (May and Baker), and veratrine B.P.C. (B.D.H.) were also used. Ethyl acetoacetate was made up in Tyrode solution or 0.9% tap water saline in order to diminish its acidity (ethyl acetoacetate *p*H 1.7, in Tyrode solution 6.7, in tap water saline 7.1). All drugs were given intravenously or into the flowmeter tubing.

RESULTS

Veratrine and Veratridine.—Veratrine and the pure alkaloid veratridine stimulate sensory receptors in both the heart and lungs (Dawes and

TABLE I

THE EFFECT OF DRUGS EXCITING CHEMOREFLEXES FROM THE HEART AND LUNGS ON PULMONARY BLOOD FLOW

The blood flow in the main pulmonary artery was measured in all experiments except those marked with an asterisk in which left pulmonary arterial blood flow was recorded. The numerals in brackets in the first column denote the number of cats used with each drug. † Denotes that the effect was sometimes diphasic (as in Fig. 3). I.v. denotes intravenous injection, i.p. injection into the pulmonary arterial tubing.

Drug	Dose and Route of Administration	Effect on Pulmonary Arterial Blood Flow (% Change)	Effect on Mean Pulmonary Arterial Pressure (mm. Hg)	Effect on Mean Systemic Blood Pressure (mm. Hg)
Veratridine (5)	15–70 μg. i.v. and i.p.	−30 to −50	−7 to −16	−34 to −86
Veratrine (3)	10–25 μg. i.p.	−15 to −60	−2 to −7	−36 to −66
Veratrine (6)*	30–100 μg. i.p.	−8 to −60	−1 to −3	−24 to −90
Phenyldiguanide (13)	100–300 μg. i.p.	−13 to −60	0 to −11	−18 to −66
2-α-Naphthylethylisothiourea (9)	75–100 μg. i.v. and i.p.	−20 to −60	−2 to −10	−36 to −74
Ethyl acetoacetate (8)	100–200 mg. i.v. and i.p.	−15 to −60†	1st phase −2 to −14 2nd phase +8 to +10	†−22 to −64

Comroe, 1954). Both substances when injected either intravenously or into the pulmonary flowmeter circuit caused a profound diminution in pulmonary blood flow, and a fall in mean pulmonary arterial pressure (Table I and Fig. 1). The increase in pulmonary arterial pulse pressure has also been demonstrated with a condenser manometer and may be due to increased stroke volume consequent upon bradycardia. There was usually a slight rise in left atrial pressure. These effects were abolished by section of the vago-sympathetic trunks in the neck.

Measurements of pulmonary vascular resistance before and after administration of veratrine in 7 cats showed very slight changes in either direction (Table II).

Amidine Compounds.—A large number of these compounds give rise to reflexes originating in the heart and in the lungs (Dawes, Mott and Widdicombe, 1951). The two compounds investigated were phenyldiguanide and 2-α-naphthylethyl*iso*thiourea, both of which caused a fall in cardiac output, an increase in pulmonary pulse pressure (observed in condenser manometer records) a rise in left atrial pressure, and, usually, a slight fall in mean pulmonary arterial pressure (Table I, Fig. 2). These effects were abolished by section of the cervical vago-sympathetic trunks.

An attempt was made to find out whether the fall in cardiac output after these substances was due to diminished venous return following peripheral vasodilatation, bradycardia, or increased pulmonary vascular resistance.

Experiments in which a Starling resistance was inserted into the main pulmonary trunk and the lungs were perfused at constant pressure showed that the fall in cardiac output was not directly due to increased pulmonary vascular resistance. Injection of phenyldiguanide in six such preparations still caused a fall in *right ventricular output.* To test the importance or otherwise of peripheral vasodilatation, the fall in arterial pressure was minimized in four cats by the insertion of a pressure stabilizer into the abdominal aorta. The fall in blood pressure after injection of phenyldiguanide was greatly reduced by this procedure, but the fall in cardiac output was as great or greater

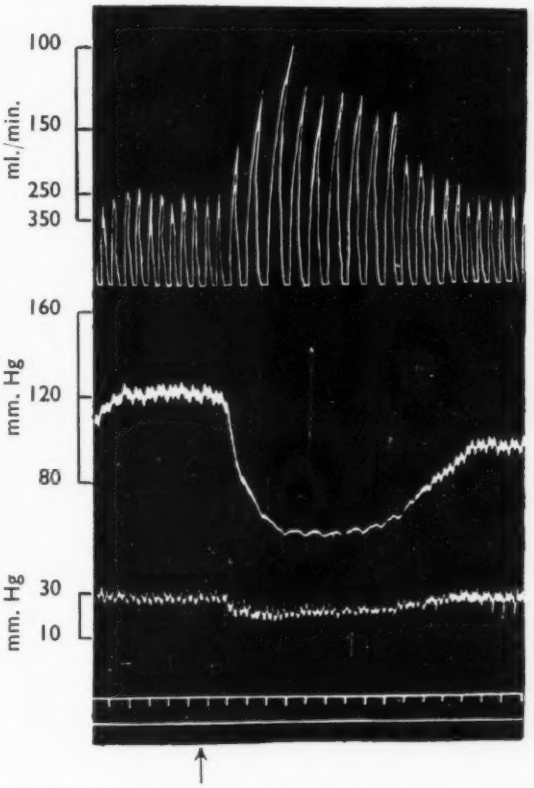

FIG. 1.—Cat (3·1 kg.). Chloralose anaesthesia. Records from above downwards, pulmonary blood flow, carotid arterial pressure, pulmonary arterial pressure and time, 10 sec. Veratrine (10 μg.) was injected at the arrow into the distal pulmonary cannula.

TABLE II

THE EFFECT OF VERATRINE ON PULMONARY VASCULAR RESISTANCE

30 to 100 μg. of veratrine was injected into the pulmonary arterial tubing. "Before" and "After" refer to measurements made before and after the injection of veratrine. Where successive measurements are given, these were made at corresponding times for flow pressure and resistance estimations.

Expt. No.	Left Pulmonary Arterial Blood Flow (ml./min.)		Mean Pulmonary Arterial Pressure (mm. Hg)		Mean Left Atrial Pressure (mm. Hg)		Resistance P.R.U. (mm. Hg/ml./min.)	
	Before	After	Before	After	Before	After	Before	After
1 (a)	56	48, 55	26	26, 24	8	8, 8	0·32	0·37, 0·29
(b)	54	32, 31	24	22, 19	7	7, 8	0·31	0·47, 0·36
2	47	38, 35, 37	17	16, 16, 14	2	3, 3, 3	0·32	0·34, 0·37, 0·3
3	50	36, 34	18	16, 15	2	2, 1	0·32	0·39, 0·41
4	51	50, 52, 47	25	25, 24, 25	3	3, 4, 4	0·43	0·44, 0·38, 0·45
5	27	24, 24	30	22, 29	5	5, 6	0·93	0·71, 0·96
6	50	48, 36, 38	17	17, 15, 14	4	4, 4, 4	0·26	0·27, 0·31, 0·26
7	42	33	27	22	3	5	0·57	0·52

than before. Both right and left atrial pressures rose considerably, so it is probable that an adequate venous return was maintained. In five cats, atropine (1 mg./kg.) was given to abolish the bradycardia. There was then little or no fall in cardiac output on administration of phenyldiguanide, and the fall in blood pressure was reduced to only 10 to 24 mm. Hg.

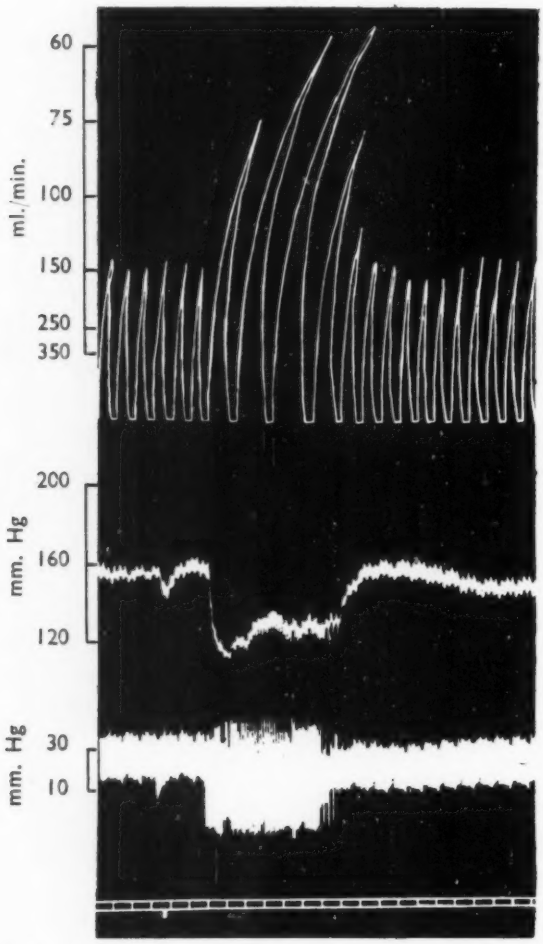

FIG. 2.—Cat (2·1 kg.). Chloralose anaesthesia. Records as in Fig. 1. 30 μg. of 2-α-naphthylethyl*iso*thiourea was injected into the distal pulmonary cannula at the signal mark.

Diphenhydramine. — Diphenhydramine and other antihistamines elicit reflexes from the lungs of the cat similar to those caused by amidines (Jones, 1952). In the dog, the two classes of compound act differently (Dawes, Mott, and Widdicombe, 1952) and in the cat differences were detected in their respective effects on tidal air (Barer and Nüsser, 1953). Table III shows that diphenhydramine differed from amidines and veratrine in that, although it caused a diminution in pulmonary blood flow, this was accompanied, in all but one cat, by a rise in mean pulmonary arterial pressure, and both these changes still took place after section of the vago-sympathetic trunks in the neck. In 4 isolated cat lungs perfused at constant blood flow, the pulmonary arterial pressure rose after administration of diphenhydramine in doses of 0.5 to 5 mg. There was therefore an increase in pulmonary vascular resistance. When a flowmeter was inserted into the left pulmonary artery, intravenous diphenhydramine caused first an increase and then (in 2 out of 3 experiments) a decrease in left pulmonary arterial blood flow (Table III, Expt. Nos. 5, 6 and 7). The preliminary increase was presumably due to the fact that the drug reached the right lung first and caused vasoconstriction and a temporary diversion of blood flow to the left lung before the drug reached this through the flowmeter circuit. When diphenhydramine was injected directly into the left pulmonary artery only a diminution of blood flow was observed (Table III, Expt. No. 6).

Ethyl Acetoacetate.—This substance was shown by Bagoury and Samaan (1940) to initiate reflexes from the lungs of dogs which resemble those excited by veratrine and amidines in cats. Its interest lies in its closer relationship to a physio-

TABLE III

THE EFFECT OF DIPHENHYDRAMINE ON PULMONARY BLOOD FLOW

LPA denotes left pulmonary artery; MPA, main pulmonary artery. In all experiments the bubble flowmeter was used except in Expt. Nos. 3 and 4 where the density flowmeter was employed. After the injection of the drug, the successive pulmonary blood flow measurements were made as rapidly as possible. I.v. denotes intravenous injection; i.p., injection into the pulmonary arterial tubing.

Expt. No.	Site of Flowmeter	Dose (mg.) and Route of Administration	Vagi	Pulmonary Blood Flow (ml./min.)		Effect on Arterial Pressure (mm. Hg)	
				Before Injection	After Injection	Pulmonary	Carotid
1	MPA	5 i.v.	Intact	188	36	+10, −4	−38
2	,,	5 i.v.	,,	215 207	60	+12	−66
	,,	5 i.v.	Cut	207 207	86	+ 8	−42
3	,,	5 i.p.	,,	167	117	+ 3	−36
4	,,	10 i.p.	Intact	197	86	+1, −3	−76
5	LPA	10 i.v.	,,	13 13	27 28 11 14	+28	−48
		10 i.v.	Cut	30 30 29	40 35 7 15	+20	−60
6	,,	13 i.v.	,,	43 43	42 46 55 27 29 37 44	+20	−50
	,,	10 LPA	,,	40 41	20 15 17 16 16	+ 6	−40
7	,,	10 i.v.	,,	16 16	25 38 30 21 23	+ 8	−44

logical substance (acetoacetic acid) than the other drugs which give rise to pulmonary chemoreflexes. Similar effects were produced in cats, but the doses required, 100 to 200 mg., were much higher than those used by Bagoury and Samaan (1940) in dogs. In cats, intravenous injection of ethyl acetoacetate caused a fall in blood pressure, arrest of respiration, sometimes rapid shallow breathing and occasionally a diminution in tidal air. That the effect on respiration was a reflex due to receptors in the lungs was shown by the fact that there was no change in breathing when ethyl acetoacetate was injected into the left atrium, or after intravenous injection when the vago-sympathetic trunks had been cut. This pulmonary respiratory reflex resembled the pulmonary amidine reflex in that it was not abolished until the cervical vagi were cooled to 0 to 2.5°, which is a much lower

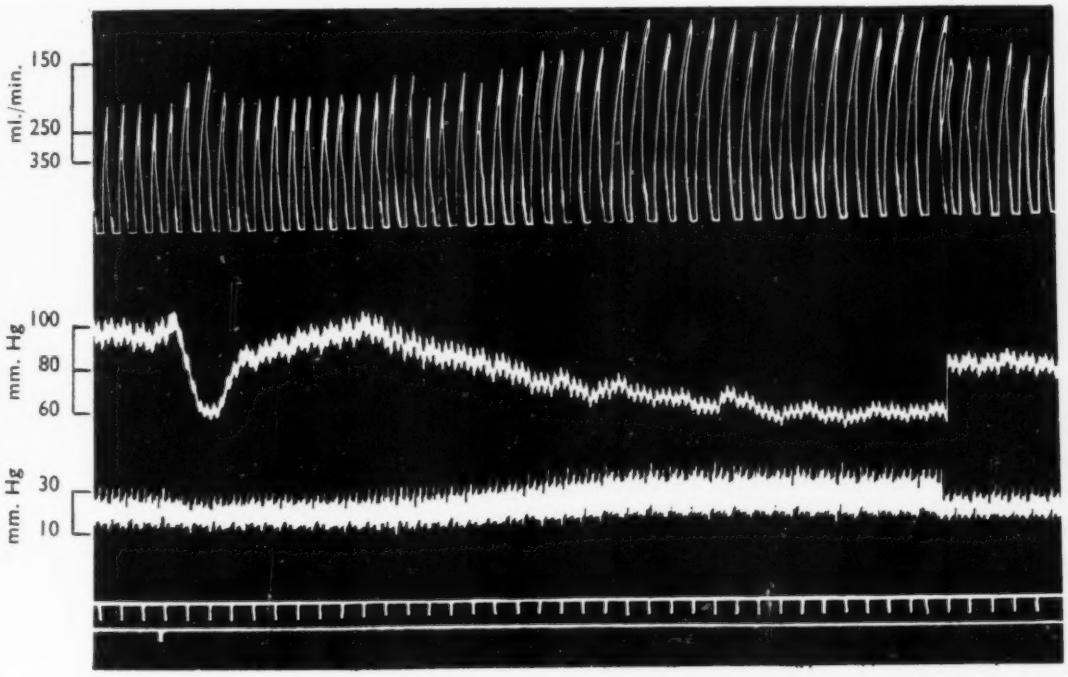

FIG. 3.—Cat 3.0 kg. Chloralose anaesthesia. Records as in Fig. 1. Ethyl acetoacetate (150 mg.) was injected into the distal pulmonary cannula at the signal mark. Partial recovery after an interval of 14 min. is shown on the right.

temperature than that required to inactivate the pulmonary stretch receptors (Dawes and Comroe, 1954). The effect of ethyl acetoacetate on cardiac output and pulmonary arterial pressure was complex (Table I and Fig. 3). There was always a fall in cardiac output, but this was frequently in two phases. The first phase was accompanied by a slight fall or no change and the second by a rise in mean pulmonary arterial pressure, and all these effects were still present after section of the vago-sympathetic trunks. The solution of ethyl acetoacetate was nearly saturated and slightly unstable, so that the two liquids occasionally separated. It was therefore considered possible that the second phase might be due to this effect happening *in vivo,* the droplets of ethyl acetoacetate acting as minute emboli. For comparison chlorbismol was injected in 4 vagotomized cats as a means of producing emboli, and caused a decline in cardiac output and a rise in pulmonary arterial pressure similar to that following administration of ethyl acetoacetate.

Discussion

All the substances tested produced a fall in cardiac output, accompanied either by a fall (veratrine, amidines, ethyl acetoacetate, first phase) or a rise (diphenhydramine ; ethyl acetoacetate, second phase) in mean pulmonary arterial pressure. The actions of veratrine and the amidines were abolished by section of the vago-sympathetic trunks in the neck, while those of diphenhydramine and ethyl acetoacetate were not. The known features of the pulmonary and cardiac chemoreflexes are therefore a fall in blood pressure, bradycardia, arrest of respiration or rapid shallow respiration, a fall in cardiac output, frequently a decrease in tidal air, an increase in left atrial pressure, and variable changes in pulmonary arterial pressure.

The small and variable changes in pulmonary vascular resistance following veratrine suggest that it has no important direct or reflex effect on the diameter of the pulmonary vascular bed. The small changes seen were more likely due to passive effects on vessel diameter caused by the decrease in flow, or to the rise in left atrial pressure which sometimes follows its administration. Thus Green, Lewis, Nickerson, and Heller (1944) have shown that there is a non-linear relationship between pressure and flow in certain regions, and therefore an alteration in resistance, which is probably due to a passive expansion of the vessels at high rates of flow and a diminution in their size at low rates of flow. Such non-linear pressure/flow curves have been demonstrated in the lungs of dogs by a number of workers and in isolated perfused cat lungs (Carlill, Duke and Jones, 1957). Similarly in cats with the thoracic contents exposed to the atmosphere in which a flowmeter was inserted into the left pulmonary artery, although there was a linear relationship between pressure and flow over a wide range of variation in flow, this straight line, when extrapolated, cut the axis at a positive pressure probably representing the critical closing pressure (Barer and Nüsser, 1957). The resistance as calculated from the ratio pressure/flow therefore increased as blood flow decreased. Carlill and Duke (1956) showed that raising the left atrial pressure in isolated perfused cat lungs led to a considerable reduction in pulmonary vascular resistance, especially when the left atrial pressure was less than 15 cm. saline. Although it was possible that second order reflexes, such as those described by Daly and Daly (1957a and b), might be initiated by the changes in systemic pressure which these substances produce, and thereby lead to changes in pulmonary vascular resistance, there is, as stated above, no *prima facie* evidence of such changes.

The similarity between the changes in the pulmonary vascular system after amidines and veratrine suggests that the former compounds have also no important direct or reflex action on the pulmonary vessels. Preliminary measurements of resistance after 2-α-naphthylethyl*iso*thiourea (not included in this report because only substituted values for the left atrial pressure were used) showed very small changes in either direction as after veratrine.

Since diphenhydramine and ethyl acetoacetate in the second phase of their action caused a fall in pulmonary blood flow and a rise in pulmonary arterial pressure which persisted after section of the vago-sympathetic trunks, they may have had a direct constrictor effect on the pulmonary vascular tree similar to that caused by 5-hydroxytryptamine (Ginzell and Kottegoda, 1953), which also elicits pulmonary chemoreflexes. Experiments on perfused lungs showed this to be true for diphenhydramine. Diphenhydramine has probably also a direct effect on bronchial muscle (Barer and Nüsser, 1953), which might in itself increase the pulmonary vascular resistance indirectly (Daly and Hebb, 1941). The results obtained with diphenhydramine also emphasize the need for caution in interpreting the effects of drugs on the pulmonary circulation when the flow through only one lung is measured.

The fall in cardiac output which accompanied all the reflexes investigated could have been due to increased pulmonary vascular resistance, diminished venous return, bradycardia, or a combination of these factors. Increased pulmonary vascular resistance can be ruled out as a cause because only after diphenhydramine and ethyl acetoacetate was there a rise in pulmonary arterial pressure, and this was certainly not sufficient to cause right heart failure. For amidines, the most thoroughly investigated substances, it was shown that the right heart output declined even when the output resistance was constant. When the arterial pressure was maintained with a compensator, the right heart output was still reduced, but when the bradycardia was abolished by atropine, a procedure which may also have affected the venous return, there was no longer any significant fall in cardiac output. Taken together, these results suggest that the bradycardia is probably the most important if not the sole factor responsible for the diminished right heart output when these reflexes are excited.

We wish to thank Dr. G. S. Dawes for his help and criticism and Mr. W. A. Ryder for technical assistance.

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THE PHARMACOLOGICAL ACTIONS OF SOME MUREXINE-LIKE SUBSTANCES

BY

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Four choline esters of imidazole acids, two imidazole ethers of choline and thirteen ring-substituted murexine-like compounds were compared with murexine for their muscle-paralysing and their nicotine-like effects. Dihydromurexine appeared, in animal experiments, to be the most potent derivative, but it was shown to be less effective than murexine in man. Among the other compounds, imidazolebutyrylcholine and imidazolepropoxycholine appeared to be worthy of particular consideration. The relation between the chemical structure of the murexine-like substances studied and their pharmacological effects is discussed.

The main pharmacological properties of murexine (urocanoylcholine, [2-β-imidazol-4′(5′)-ylacryloyloxyethyl]trimethylammonium chloride hydrochloride), a naturally occurring neuromuscular blocking agent found in the hypobranchial body of some prosobranchiate molluscs, have been described in previous papers by ourselves (Erspamer and Glässer, 1957) and others (Tabachnick and Roth, 1957; Quilliam, 1957; Winbury, 1957; Keyl and Whittaker, 1958).

The investigations described below are concerned with some pharmacological effects of 19 murexine-like derivatives, synthesized by Pasini, Vercellone, and Erspamer (1956) and by Pasini and Coda (1957). Four are murexine analogues (choline esters of imidazole acids), two are imidazole ethers of choline, and 13 are choline esters of acrylic or propionic acids substituted in the β-position with heterocyclic rings different from the imidazole ring.

A few comparative experiments were also carried out with $\beta\beta$-dimethylacryloylcholine or senecioylcholine, a new choline ester recently discovered by Whittaker (1957) in the marine gastropod *Thais floridana*.

METHODS AND MATERIALS

Murexine Samples.—Synthetic murexine chloride hydrochloride was used throughout the experiments for comparison. Murexine samples were always synthesized shortly before use because the activity of crystalline murexine chloride hydrochloride has been observed to decrease slowly on standing, apparently by as much as 50%. This puzzling fact, not yet fully elucidated by the studies of Pasini and Coda (1957), of course impairs the accuracy of the comparison of murexine with the other substances. The absolute and relative values reported in this paper should, for this reason, be regarded as only approximate. As it was not known whether the derivatives, like murexine, lost activity on standing, they were all freshly synthesized before use.

Pharmacological Methods.—The pharmacological methods used in the present investigation were identical with those previously described (Erspamer and Glässer, 1957).

Neuromuscular blocking action was tested in the intact animal and on the cat sciatic nerve-gastrocnemius muscle preparation, prepared in the manner described by Burn (1950). The doses required to cause head-drop in 50% of rabbits and to paralyse 50% of dogs were determined after rapid intravenous injection. In the cat sciatic nerve-gastrocnemius muscle preparation, maximal stimuli (rectangular current pulses of 7 msec. duration) were applied to the sciatic nerve through a unipolar electrode at a rate of 5 or 10/min. and the contractions were recorded semi-isometrically. Cats were anaesthetized with chloralose (70 mg./kg., i.v.) and the injections were given into the femoral vein. Blood pressure was recorded from the carotid artery. Respiration was recorded by means of a tracheal cannula connected to a tambour. Adequate oxygenation of the blood during apnoea was maintained by a respiratory pump.

Rabbits received intravenous injections into the ear marginal vein, mice into the tail vein.

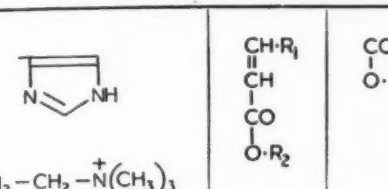
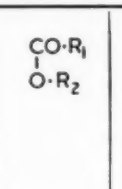
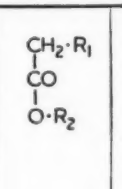
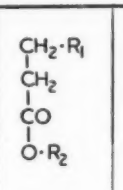
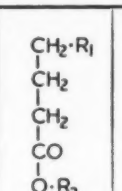
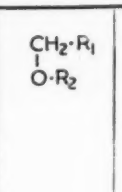
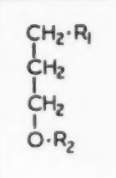
Muscarine-like action was tested on the rabbit intestine and on the rabbit atrium. The isolated frog rectus abdominis muscle and the blood pressure of

TABLE I

COMPARISON OF ACTIVITY OF MUREXINE AND OF SOME IMIDAZOLE ESTERS AND IMIDAZOLE ETHERS OF CHOLINE

Activity of murexine: 100. Dose (mg./kg. body weight of free base) shown in parentheses. The activities of the other drugs are expressed as percentages.

I, Murexine; II, imidazolecarboxylcholine; III, imidazoleacetylcholine; IV, dihydromurexine; V, imidazolebutyrylcholine; VI, imidazolemethoxycholine; VII, imidazolepropoxycholine.

	 (I)	 (II)	 (III)	 (IV)	 (V)	 (VI)	 (VII)
Mouse: LD50 (i.v.)	100 (6·45)	37 (17·0)	170 (3·8)	115 (5·57)	80 (7·9)	430 (1·5)	290 (2·24)
Rabbit: ED50 (head drop)	100 (0·50)	1·7 (29·0)	25 (2·0)	450 (0·11)	165 (0·3)	26 (1·95)	58 (0·85)
Dog: ED50 (paralysing dose)	100 (0·27)	3·5 (7·4)	67 (0·4)	1,300 (0·022)	—	—	—
Cat: sciatic nerve-gastrocnemius preparation (90 to 100% twitch depression) ..	100	3	62	330	130	25	95
Spinal cat: blood pressure	100	43	25	420	37	—	<20
Frog: rectus abdominis	100	0·8	40	700	35	0·25	1·7

the spinal cat prepared as described by Burn (1950) were used to test for nicotinic effects.

Murexine-like Compounds.—The following salts of murexine-like compounds were used in our experiments: dihydromurexine dipicrate, imidazolecarboxylcholine chloride hydrochloride, imidazoleacetylcholine dipicrate, imidazolebutyrylcholine dipicrate, imidazolemethoxycholine dipicrate, imidazolepropoxycholine dipicrate; β-pyrid-2-ylacryloylcholine picrate, β-pyrid-2-ylpropionylcholine picrate, β-pyrid-4-ylacryloylcholine dipicrate, β-pyrid-4-ylpropionylcholine dipicrate, β-pyrimidin-2-ylacryloylcholine picrate, β-pyrimidin-4-ylacryloylcholine dipicrate, β-pyrimidin-4-ylpropionylcholine dipicrate, β-pyridazin-3-ylacryloylcholine picrate, β-pyridazin-3-propionylcholine picrate, β-thiazol-2-ylacryloylcholine picrate, β-thiazol-2-ylpropionylcholine picrate, β-fur-2-ylacryloylcholine chloride, β-fur-2-ylpropionylcholine picrate.

Weights of the above compounds were always quoted in terms of their free bases.

RESULTS

Imidazole Esters and Imidazole Ethers of Choline

Table I shows the relative potency of the six new compounds compared with that of murexine. Some absolute values, expressed in mg. free base/kg. body weight, are given in parentheses.

In Table II the paralysing doses in the cat gastrocnemius preparation of murexine (I) are compared with those of the other compounds.

Other pharmacological effects of the substances examined are as follows:

Imidazoleacetylcholine.—Like murexine, imidazoleacetylcholine (2-imidazol-4′(5′)-ylacetoxyethyltrimethylammonium) elicited variable blood pressure responses in the intact dog, cat, and rabbit, following rapid intravenous injection. However, a more or less marked hypertension was predominant after moderate and high doses of the

TABLE II

COMPARISON OF PARALYSING ACTION OF MUREXINE AND OTHER MUREXINE-LIKE COMPOUNDS ON CAT SCIATIC NERVE GASTROCNEMIUS MUSCLE PREPARATION

Numerals in bold type indicate minimum dose required to cause 90 to 100% twitch reduction. The roman numerals refer to the structural formulae in Table I. The doses are expressed in terms of the free base.

Dose (mg./kg.)	Maximum Twitch Reduction (Complete Inhibition: 100%)	Time for 50% Recovery (min.)
Murexine (I)		
0·075	31	3
0·15	74	3·45
0·225	96	8·50
0·375	100	15
0·750	100	33
Imidazolecarboxylcholine (II)		
4·0	40	3
8·0	95	7
Imidazoleacetylcholine (III)		
0·042	25	0·30
0·084	60	1
0·17	85	1·30
0·34	90	1·30
1·70	100	5
Dihydromurexine (IV)		
0·018	46	1·40
0·035	71	2·10
0·07	96	3·20
0·105	99	3·22
0·140	100	4
0·35	100	9
Imidazolebutyrylcholine (V)		
0·035	18	0·10
0·105	81	1·10
0·175	98	3
0·70	100	4
Imidazolepropoxycholine (VI)		
0·06	31	1·40
0·12	78	5
0·18	88	10
0·24	97	15

drug (0.4 to 4 mg./kg.). The pressure rise was sometimes preceded by a transient fall, often accompanied by bradycardia. We observed, in accord with Winbury (1957) and Roth, Rubin, Tabachnick, and Govier (1958), that hexamethonium (2 to 3 mg./kg., i.v.) reduced or abolished only the hypertension, whereas atropine (1 mg./kg., i.v.) eliminated the fall of blood pressure and heart rate. On the other hand, eserine salicylate (50 μg./kg., i.v.) enhanced the rise of pressure in the spinal cat, the size of which (Table I and Fig. 1) was approximately one quarter of that produced by murexine. Similarly, eserine enhanced and prolonged the neuromuscular block exerted by imidazoleacetylcholine in the cat gastrocnemius preparation (Fig. 3).

The rabbit atrium was little affected by this choline ester. Doses of 50 μg./ml., and above, caused only a slight increase in the amplitude and a moderate decrease in the frequency of contraction. Imidazoleacetylcholine elicited a weak contraction of the rabbit colon which was partially antagonized by atropine sulphate (5×10^{-8}). The minimum effective dose was approximately 2 μg./ml.

The minimum concentration of imidazoleacetylcholine active on the leech muscle was 10^{-5}. Previous treatment with eserine caused a fourfold increase in sensitivity and, after eserine, the contracture curve did not reach a plateau so readily as before.

Dihydromurexine (Imidazolepropionylcholine). —The action of dihydromurexine (2-β-imidazol-4′(5′)-ylpropionyloxyethyltrimethylammonium) on

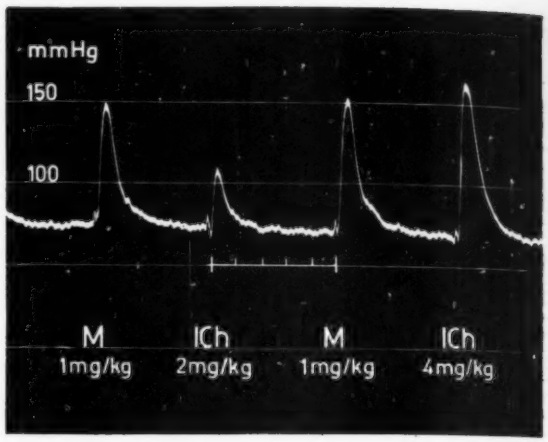


FIG. 1.—Blood pressure of spinal cat. Pressure rise caused by murexine (M) in this experiment was approximately 3·4 times greater than that caused by imidazoleacetylcholine (ICh). Time, 1 min.

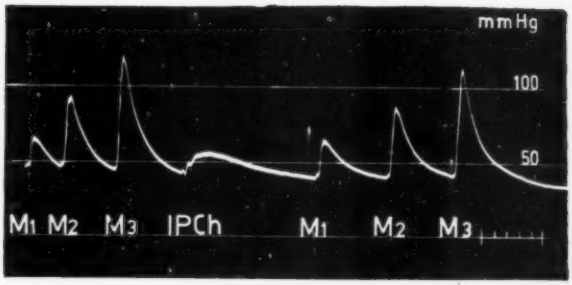


FIG. 2.—Blood pressure of spinal cat. M1, M2, M3 refer to 1, 2, 3 mg./kg. of murexine respectively; IPCh, 5 mg./kg. of imidazolepropoxycholine. Pressure rise caused by 1 mg./kg. murexine was greater, although of shorter duration, than that elicited by 5 mg./kg. imidazolepropoxycholine. Time, 1 min.

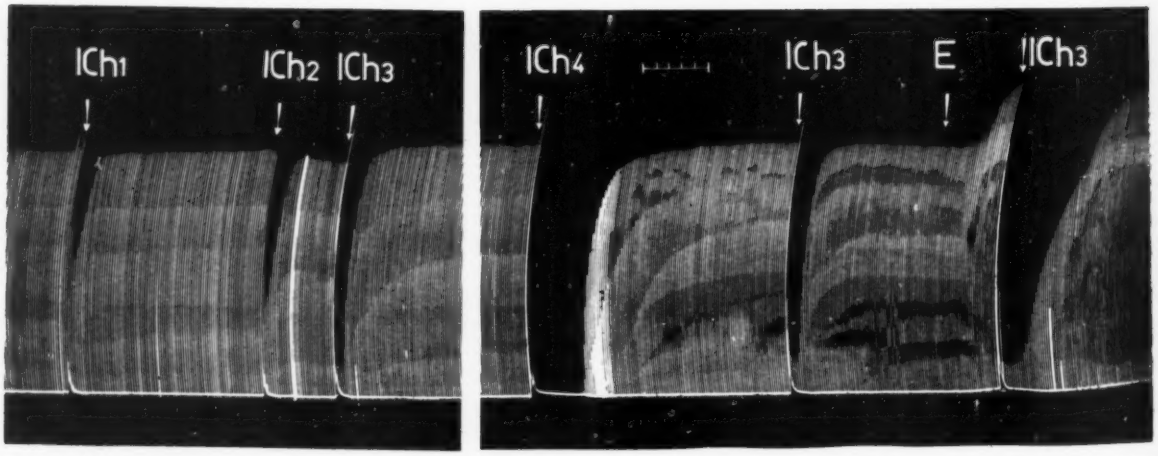


FIG. 3.—Cat sciatic nerve-gastrocnemius muscle preparation. ICh1, ICh2, ICh3, ICh4 represent 0.2, 0.4, 1, 5 mg./kg. of imidazoleacetylcholine respectively; E, 50 μg./kg. of eserine salicylate. All injections were given intravenously. Neuromuscular block, sometimes preceded by increased twitch height, was always short-lasting. Eserine prolonged paralysis markedly. Time, 1 min.

the blood pressure resembled that of murexine and imidazoleacetylcholine. Like Conroy, Kappell, Ferruggia, and Randall (1956), we have found that dihydromurexine was 4 to 5 times more potent than murexine in raising blood pressure in both the cat and the dog. Depending on circumstances, the drug (3 μg./ml., or more) caused either a depression or a stimulation of the rabbit colon. The stimulation was blocked by atropine. Dihydromurexine produced no appreciable effect on the rabbit atrium, in doses up to 10 μg./ml. Other pharmacological effects of dihydromurexine

TABLE III

ACTIVITY OF RING-SUBSTITUTED MUREXINE-LIKE COMPOUNDS COMPARED WITH THAT OF MUREXINE
Activity of murexine: 100. Dose (mg. free bases/kg. body weight) shown in parentheses. The activities of the other derivatives are expressed as percentages.

R_α = $-CH{=}CH-CO-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$ (acrylic series) R_β = $-CH_2-CH_2-CO-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$ (propionic series)		Mouse LD50 (i.v.)	Rabbit Head Drop ED50	Cat Sciatic Nerve Gastrocnemius Muscle	Spinal Cat Blood Pressure	Frog Rectus Abdominis
Pyrid-2-yl-	R_a	52 (12·7)	110 (0·45)	100	300	800
	R_β	<30 (>20)	<30 (>1·5)	90	—	1,400
Pyrid-4-yl-	R_a	—	—	<10	15	40
	R_β	—	—	—	—	130
Pyrimidin-2-yl-	R_a	—	—	<5	<10	<5
Pyrimidin-4-yl-	R_a	<30 (>20)	—	40	—	115
	R_β	57 (11·3)	—	125	—	750
Pyridazin-3-yl-	R_a	23 (28·5)	62 (0·8)	20	300	200
	R_β	38 (17·0)	<50 (>1·0)	75	350	900
Thiazol-2-yl-	R_a	—	<30 (>1·7)	10	—	60
	R_β	—	<35 (>1·5)	10	—	10
Fur-2-yl-	R_a	—	13 (3·8)	10	—	20
	R_β	—	<25 (>1·8)	10	—	75
$\beta\beta$-Dimethylacryloylcholine		—	—	60	—	200

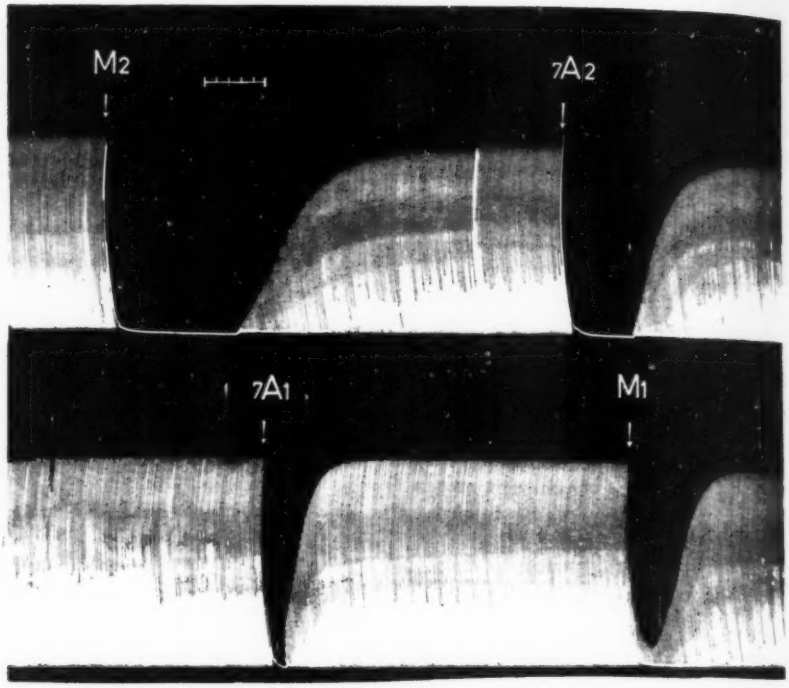


FIG. 4.—Cat sciatic nerve-gastrocnemius muscle preparation. 7A1, 7A2 refer to 0.3 and 1.2 mg./kg. of β-pyridazin-3-ylacryloylcholine respectively; M1, M2 represent 0.2, 0.4 mg./kg. of murexine respectively. Block caused by β-pyridazin-3-ylacryloylcholine was much shorter than that produced by murexine. Time, 1 min.

on the superior cervical ganglion of the cat, on the femoral blood flow of the dog, and on the histamine-induced contraction of the intestinal and tracheal smooth muscle of the guinea-pig have been recently described by Kewitz (1955) and Conroy *et al.* (1956), by Winbury, Wolf, and Tabachnick (1957) and Winbury (1957), and by Tabachnick and Roth (1957), respectively.

Imidazolecarboxylcholine and Imidazolebutyrylcholine.—Both drugs caused a small contraction of the isolated rabbit colon (minimum active dose 2 to 3 μg./ml.). Atropine hardly influenced the action of imidazolecarboxylcholine (2-imidazol-4′(5′)-ylcarbonyloxyethyltrimethylammonium), but effectively blocked that of imidazolebutyrylcholine (2-γ-imidazol-4′(5′)-ylbutyryloxyethyltrimethylammonium). On the rabbit atrium the drugs were ineffective in doses up to 50 μg. and 10 μg./ml., respectively.

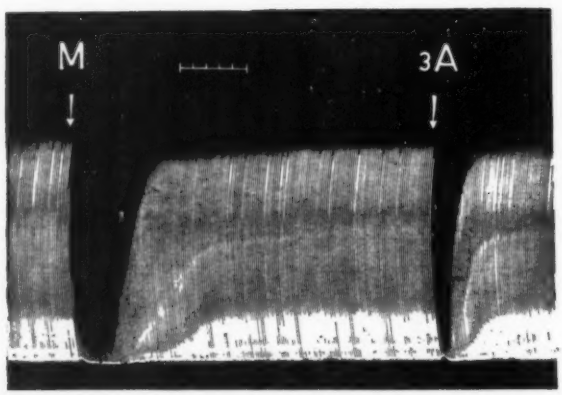


FIG. 5.—Cat sciatic nerve-gastrocnemius muscle preparation. 250 μg./kg. of murexine (M) and 250 μg./kg. of β-pyrid-2-ylacryloylcholine (3A) produced twitch depression of same magnitude. The duration of neuromuscular block was considerably longer for murexine. Time, 1 min.

Imidazolemethoxycholine and Imidazolepropoxycholine.—The minimum dose of both drugs which stimulated the isolated rabbit colon was approximately 2 to 3 μg./ml. Atropine (5×10^{-8}) caused an almost total inhibition of the effect of imidazolepropoxycholine [2-(3-imidazol-4′(5′)-ylpropoxy)ethyltrimethylammonium], but only a 50% inhibition of that of imidazolemethoxycholine (2-imidazol-4′(5′)-ylmethoxyethyltrimethylammonium). In concentrations up to 20 μg./ml. both drugs had little effect on the rabbit atrium. The only action of imidazolepropoxycholine was to cause a slight reduction in the frequency of contraction.

Ring-substituted Murexine-like Compounds

Table III shows the relative potency, on a molar basis, of the ring-substituted compounds, with respect to murexine.

The duration of the neuromuscular block was very short for all substances in this series. Following abolition of the twitch, 50% recovery

was generally observed in 2 to 3 min. (Figs. 4 and 5). Hence, the compounds are rather easily inactivated in the organism.

On the rabbit atrium, both the pyrimidin-4-yl derivatives were ineffective up to concentrations of 20 μg./ml.; β-pyrid-2-ylacryloylcholine showed, at the same dosage, a slight negative chronotropic effect and, occasionally, a slight positive inotropic effect.

Keyl, Michaelson, and Whittaker (1957) examined other ring-substituted choline esters and found that, on the frog rectus preparation, indolylacetylcholine manifested the same stimulant effect as murexine, indolylpropionylcholine 60%, and nicotinylcholine 10% of the effect of murexine. Our investigations confirm their statement that ββ-dimethylacryloylcholine has a very strong nicotinic action, in addition to its remarkable muscle-paralysing effect.

Discussion

The experiments described in this paper were carried out with the main purpose of throwing some light on the problem of the relation between the chemical structure and pharmacological actions of murexine derivatives.

Concerning the first series of the murexine-like compounds examined (the imidazole esters and imidazole ethers of choline) the following conclusions may be drawn from the experimental results:

(*a*) Hydrogenation of the acryloyl side-chain into propionyl caused, as a rule, a powerful increase in both nicotinic and neuromuscular blocking actions. In rabbits, cats, and dogs dihydromurexine was as active as, or even more active than, suxamethonium in producing paralysis of the skeletal musculature. On the frog rectus abdominis dihydromurexine was nearly as potent as acetylcholine in causing contracture.

A remarkable exception is man, for whom the drug is less active than murexine as a muscle-relaxant agent. In fact, in preliminary experiments carried out by De Blasi and Leone (personal communication), dihydromurexine, given by slow intravenous injection in doses up to 1 g., failed to produce consistent muscular relaxation. The reason for this has been explained, at least in part, by the observations of Foldes, Erdös, Baart, and Shanor (1957) and Grelis and Tabachnick (1957), who demonstrated that, whereas murexine is slowly hydrolysed by human plasma cholinesterase, the rate of hydrolysis of dihydromurexine is as fast as that of acetylcholine and 18 times faster than that of murexine.

(*b*) The length of the side-chain of the imidazole acid was of critical importance. A three carbon atom chain seemed to be optimal both for maximal blocking and nicotinic potency. Imidazoleacetyl- and imidazolecarboxyl-choline on the one hand and imidazolebutyrylcholine on the other were less potent than dihydromurexine. Imidazolecarboxylcholine was the least active member of the series, having a paralysing action barely 1% of that of dihydromurexine.

(*c*) Substitution of the ester linkage by an ether linkage might produce different results. In muscle-blocking action determined on the intact rabbit and the cat gastrocnemius preparation, the propoxy derivative appeared to be 1/3 to 1/7 as active as the corresponding propionyl derivative; the methoxy derivative, on the contrary, was 8 to 15 times more active than the corresponding carboxyl derivative. As to its nicotinic effects, imidazolepropoxycholine was approximately 1/20 as effective as dihydromurexine on the blood pressure of the spinal cat (Fig. 2) and 300 to 500 times less effective on the frog rectus abdominis. Both the ethers were more toxic in the mouse than the corresponding esters; it was, however, doubtful whether peripheral respiratory arrest was the only cause of death.

(*d*) Muscarinic actions were very weak, if present, in all compounds of this series. When compared to that of acetylcholine, the spasmogenic action on the rabbit colon was less than 1/10,000 to 1/50,000, the action on the rabbit atrium less than 1%, and the vasodilator action on the dog leg approximately 1% to 2% (Winbury *et al.*, 1957 and 1958; Winbury, 1957).

From Table I it appears that there is no constant correspondence for the different compounds between their paralysing potency as assessed in one animal species and that found in another species; dihydromurexine, for example, is 13 times more active than murexine in the dog, whereas it is only 3 to 5 times more active in the rabbit and on the cat gastrocnemius preparation, and even less active than murexine in man. Differences in the rate of enzymatic hydrolysis by blood and tissue cholinesterases may, as already stated, account for some of these species differences in blocking potency.

Table II shows that the time required for 50% recovery from a given twitch reduction may vary consistently for the different compounds. In this respect, imidazoleacetylcholine (Fig. 3) and imidazolebutyrylcholine produced the shortest neuromuscular block, imidazolepropoxycholine the longest (Fig. 2).

As for the nicotinic effects, the most interesting fact is a frequent dissociation between the nicotinic action on ganglia and adrenal medulla (blood pressure of the spinal cat) and that on the neuromuscular junction (frog rectus abdominis). For example, imidazolecarboxylcholine and imidazolepropoxycholine possess 25% and 20%, respectively, of the activity of murexine on the cat blood pressure, but only 1% to 2% of that on the frog rectus abdominis.

With regard to the second series of our products (the ring-substituted murexine-like compounds), the observations summarized in Table III allow the conclusions: (*a*) that the substitution of a propionyl for the acryloyl side-chain generally causes an enhancement of both the muscle-paralysing and the nicotinic actions; (*b*) that in the more active products the heterocyclic nucleus is linked to the acylcholine chain in such a way as to present the following sequence of atoms (Pasini *et al.*, 1956); and (*c*) that the heterocyclic nucleus

is by no means necessary for the appearance of the characteristic pharmacological effects. $\beta\beta$-Dimethylacryloylcholine, the choline ester of a simple aliphatic acrylic acid, possesses at least 50% of the muscle-paralysing potency of murexine and a nicotinic effect which is twice as strong.

Another object of this work was that of seeking a product which could be employed, more advantageously than murexine, as an adjuvant in anaesthesia. In fact the advisability of adopting murexine clinically as a muscle relaxant has yet to be established.

The present investigation was virtually unsuccessful in indicating a possible substitute for murexine. Dihydromurexine, which at first seemed on the basis of animal experiments to be the most promising substitute, turned out to be practically ineffective in human beings. Unfortunately, owing to difficulties in their synthesis, imidazolebutyrylcholine and imidazolepropoxycholine could not be prepared in amounts large enough to permit a clinical trial. The substantial attenuation of the disturbing nicotinic effects on blood pressure and on skeletal musculature shown by the second substance could represent an important advantage over murexine.

The ring-substituted murexine-like compounds were not considered for clinical trial. The three or four which seemed worthy of fuller consideration presented very strong nicotinic effects, in addition to an exceedingly short duration of the muscle-paralysing action.

Gruner and Kewitz (1955) have tentatively identified with imidazoleacetylcholine an imidazole derivative found by them in extracts of mammalian brain. It is interesting to note that in our experiments, as in those of Roth *et al.* (1958), imidazoleacetylcholine proved to be one of the less potent of the imidazole derivatives tested. It should be added that Correale (1958) was unable to detect imidazoleacetic acid or imidazoleacetylcholine in brain extracts of lower vertebrates, but succeeded in demonstrating, by paper chromatography, the occurrence of enormous amounts (apparently up to 600 to 800 μg./g. fresh tissue) of another unknown imidazole derivative. This is similar, in many respects, to imidazoleacetic acid and imidazolepropionic acid and, therefore, could perhaps be identical with the imidazole compound obtained by Gruner and Kewitz (1955) following hydrolysis of their new choline ester.

We should like to thank Dr. V. P. Whittaker for a generous sample of $\beta\beta$-dimethylacryloylcholine.

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HAEMOGLOBINURIA CAUSED BY PROPYLENE GLYCOL IN SHEEP

BY

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Haemoglobinuria occurred in sheep anaesthetized by an intravenous injection of pentobarbitone sodium containing propylene glycol: an equivalent dose failed to cause haemoglobinuria in rabbits. Intravenous injection of an aqueous solution of 20% propylene glycol caused haemoglobinaemia and haemoglobinuria in sheep. Neither distilled water nor 20% glycerol in water administered under identical conditions produced these effects.

Haemoglobinuria occurred on some occasions when an aqueous 20% solution of propylene glycol was administered to sheep after an injection of saline: it never occurred when a solution of 20% propylene glycol prepared with physiological saline was injected. It is suggested that saline may protect against the haemolytic action of propylene glycol in sheep and that propylene glycol should be avoided as a menstruum for pharmaceutical preparations to be used for injection into the blood stream of these animals.

Propylene glycol has been used for some time as a vehicle for medicinal and pharmaceutical preparations because of its solvent properties and apparently low toxicity (Seidenfeld and Hanzlik, 1932; Hunt, 1932). In a pharmacological study of the effects of propylene glycol on dogs, chickens and rabbits, Lehman and Newman (1937) found that an intravenous injection of a 33.3% solution of propylene glycol in physiological saline produced haemolysis and haemoglobinuria. Hanzlik, Newman, Van Winkle, Lehman and Kennedy (1939a) reported that 25 ml. of a 50% solution of propylene glycol in saline injected intravenously at a rate of not less than 0.5 ml./kg. body weight/min. was lethal to dogs. However, McGavack and Vogel (1944) gave daily intravenous injections of propylene glycol in relatively large doses to dogs and humans without any signs of abnormal effects.

Examination of the effects on metabolic activities made by Hanzlik, Newman, Van Winkle, Lehman and Kennedy (1939b) indicates that propylene glycol can increase glycogen storage in rats. Laug, Calvery, Morris, and Woodard (1939) in a systematic study of the toxicology of glycols consider that propylene glycol can be safely tolerated in small doses by the animal body since it can be oxidized and probably converted to a normal body constituent. It thus resembles ethyl alcohol and glycerol and, as with these substances, large quantities are toxic.

Among the pharmacological preparations which contain propylene glycol are various solutions of pentobarbitone sodium used to produce hypnosis and general anaesthesia in animals. Preliminary observations indicated that such preparations could produce haemoglobinuria in sheep when used to induce general anaesthesia. A systematic study was therefore carried out to see how often haemoglobinuria followed the administration of pentobarbitone sodium to sheep, and if the use of propylene glycol in proprietary preparations of the anaesthetic contributed to the haemoglobinuria.

METHODS

Altogether 19 Merino ewes aged from 2 to 10 years in which the weights varied from 35 to 50 kg., and two male Chinchilla rabbits weighing 1.5 kg., were used. Ten observations were made on six sheep which were anaesthetized for varying periods of up to 2 hr. with proprietary solutions of pentobarbitone sodium. Four of these sheep were anaesthetized with a freshly prepared 6% (w/v) solution of pure pentobarbitone sodium in water and four others received this freshly prepared solution to which had been added propylene glycol to make a final concentration of 20% (w/v).

In most experiments anaesthesia was induced in the sheep with an initial dose of 15 ml. which contained

900 mg. pentobarbitone sodium (26.4 mg./kg. body weight) injected at an approximate rate of 3 ml./min. and maintained thereafter with an injection of 2 ml. (120 mg.) every 10 min. Because of differences in individual response, the dose and the rate of administration varied slightly. An indwelling catheter was used to obtain urine samples from the bladder.

To determine the effect of propylene glycol in the same and other unanaesthetized sheep, the glycol was prepared as a 20% (w/v) solution in water and an initial volume of 15 ml. was injected intravenously at a rate of 3 ml./min. followed by 2 ml. every 10 min. for a total period of 2 hr. The propylene glycol was purified by distillation to prevent the possibility of impurities affecting the result. Urine samples were obtained by catheter before the injection and thereafter at half-hourly intervals. The urine samples were tested qualitatively for haemoglobin by the benzidine reaction and if positive were confirmed by spectroscopy. From six of the sheep, urine samples were taken for an additional 3 hr. following cessation of the glycol injection. Blood samples from these animals were collected by venipuncture of the external jugular vein, meticulous care being exercised to prevent extravascular haemolysis. Samples of urine were obtained from the bladder at the same time. The blood was centrifuged and the amount of haemoglobin in the plasma and urine samples was determined by a modification of the method of Bing and Baker (1931) as outlined by Dacie (1956) and adapted for spectrophotometric estimation using a Unicam spectrophotometer at a wavelength of 510 λ. Haematocrit readings were determined on the blood samples from two of the animals. Tests for haemosiderin were performed on the urine samples taken during the collection period and 24 hr. later, to ascertain whether iron had been excreted in the urine as a result of reabsorption of haemoglobin by the kidney tubules.

To assess the importance of osmotic effects in the blood stream, the propylene glycol was injected at the same rate into two sheep as a 20% (w/v) solution in physiological saline (0.85%) instead of water, and an equivalent volume of distilled water was injected in the same manner as the propylene glycol into two other sheep. In addition an initial volume of 150 ml. physiological saline was given to four sheep 5 min. before the injection of 20% propylene glycol in water and the effect of this priming saline injection was noted.

The effect of administering glycerol (which like propylene glycol is a polyhydric alcohol) and ethyl alcohol was also observed in two sheep. The animals received no food or water for the period during which the urine samples were obtained.

Quantitative experiments on haemolysis were carried out *in vitro* on 10 ml. samples of blood, carefully withdrawn from the external jugular veins of four sheep, and added with gentle agitation to dry glass centrifuge tubes which all contained heparin and equivalent amounts of physiological saline, distilled water, 20% glycerol in water, 20% propylene glycol in water, 20% propylene glycol in physiological saline or physiological saline followed after 5 min. by 20% propylene glycol in water. The volumes of the respective solutions, to which the samples of blood were added, were calculated from the amount of propylene glycol usually required to produce haemoglobinuria in a sheep with a blood volume of 3.5 l.

Two rabbits were deeply anaesthetized with the same proprietary preparations of pentobarbitone sodium as used for the sheep. The anaesthetic was injected into the marginal ear vein and the urine was analysed for haemoglobin during the subsequent 4 to 5 hr.

Results

Pentobarbitone Sodium.—Eight out of ten observations made on six sheep which received proprietary preparations of pentobarbitone sodium intravenously indicated that there was haemoglobin in the urine. In the other two it is possible that insufficient pentobarbitone sodium was injected to produce the effect.

Haemoglobinuria was also observed in all the animals which received a freshly prepared 6% solution of pentobarbitone sodium containing 20% propylene glycol. There was no haemoglobinuria when propylene glycol was omitted from this solution.

Two rabbits anaesthetized with the same proprietary preparations of pentobarbitone sodium as the sheep showed no haemoglobinuria.

Propylene Glycol (20% in Water).—Haemoglobinuria with concurrent intravascular haemolysis was usually produced when 15 ml. of a solution of 20% propylene glycol in water followed by 2 ml. every 10 min. was administered to sheep as a slow steady intravenous injection. The amount of haemoglobin in the plasma was always increased in the sample taken 30 min. after the initial injection of propylene glycol, and this increase continued until the glycol injection was discontinued. Thereafter the plasma haemoglobin gradually decreased until the pre-injection value was reached about 5 hr. after the initial injection of propylene glycol.

Haemoglobin usually appeared in the urine when the plasma haemoglobin began to increase, but sometimes this effect was delayed. On these occasions haemoglobinuria did not appear until 60 to 90 min. after the propylene glycol injection had commenced. The haemoglobin had disappeared from the urine at the end of 5 hr. in all but two animals, and only small amounts remained in the urine of the latter.

The concentrations of haemoglobin in the plasma and urine of five sheep after the administration of the propylene glycol are shown in Table I. One sheep, No. 56, to which 20% propylene glycol was administered as described in Table I showed no signs of haemoglobinuria even though there was some haemolysis. A subsequent injection of the same quantity of 30% propylene glycol increased the haemolysis and haemoglobin then appeared in the urine.

Haemosiderin was not detected in the urine either during the period when haemoglobin was present or 24 hr. later: it thus appears that tubular reabsorption of haemoglobin by the kidney tubules may not have occurred during this period.

Graphs showing the haemoglobin concentrations in the plasma and urine of sheep No. 04, together with the concurrent haematocrit determinations, are given in Fig. 1. It can be seen from these curves that the degree of haemolysis and haemoglobinuria varies inversely with the ratio of blood cells to plasma, an indication of intravascular haemolysis as a result of the propylene glycol administration.

Apart from the resulting haemoglobinuria, the sheep did not appear to be affected unduly by the propylene glycol.

Propylene Glycol (20% in Saline).—Propylene glycol, when injected into sheep as a 20% solution prepared in physiological saline and administered in the same manner as 20% propylene glycol in water, never produced haemoglobinuria.

Propylene Glycol (20% in Water) after Physiological Saline.—The results of the same quantities of 20% propylene glycol in water administered 5 min. after an intravenous injection of physiological saline were varied. Two sheep which received 150 ml. physiological saline before the glycol exhibited no haemoglobinuria, while the urine of one contained haemoglobin after the severe diuresis, which followed the injection of the saline, had ceased. Haemoglobinuria without diuresis appeared in the other animal that received only 100 ml. physiological saline.

Water, Glycerol or Ethyl Alcohol.—An intravenous injection of distilled water, glycerol solution (20% in water) or ethyl alcohol (10% in water), when given under the same conditions as the 20% propylene glycol in water, did not produce haemoglobinuria in any sheep. The results of administering different solutions are summarized in Table II.

TABLE II

APPEARANCE OF HAEMOGLOBIN IN BLOOD AND URINE OF SHEEP DUE TO INITIAL INTRAVENOUS INJECTION OF 15 ML. OF VARIOUS SOLUTIONS AT 3 ML./MIN. FOLLOWED BY 2 ML. EVERY 10 MIN. FOR 2 HR.

Treatment	No. of Experiments	Haemoglobinaemia	Haemoglobinuria
Proprietary solutions of pentobarbitone sodium	10	8	8
Pentobarbitone sodium—6% (w/v) in water	4	—	—
Pentobarbitone sodium—6% (w/v) } in water	4	4	4
Propylene glycol—20% (w/v) }			
,, ,, —20% (w/v) in water	6	6	5
,, ,, —20% (w/v) in 0·85% saline	2	—	—
Propylene glycol—20% (w/v) in water after 150 ml. 0·85% saline	4	2	2
Distilled water	2	—	—
Glycerol—20% (w/v) in water	2	—	—
Ethyl alcohol—10% (v/v) in water	2	—	—

In vitro *Experiments.*—Little information was gained from adding freshly and carefully collected blood to solutions of propylene glycol, saline and glycerol. There was haemolysis in only half the samples obtained when blood was added to an aqueous solution of 20% propylene glycol, but when blood was mixed with saline and then added to the propylene glycol solution haemolysis always occurred. Haemolysis was not detected when blood was mixed with saline, with glycerol or with a 20% solution of propylene glycol in saline.

TABLE I

HAEMOGLOBIN CONCENTRATIONS IN PLASMA AND URINE OF SHEEP

The initial injection of 15 ml. of propylene glycol was followed by 2 ml. every 10 min. for 2 hr.

Animal No.	Weight (kg.)	Conc. of Propylene Glycol in Water (%)	Haemoglobin in Plasma (g./100 ml.) Hr. after Initial Injection								Haemoglobin in Urine (g./100 ml.) Hr. after Initial Injection							
			0	½	1	1½	2	2½	3	5	0	½	1	1½	2	2½	3	5
52	42	20	·01	·16	·24	·45	·55	·42	·21	·06	—	—	·14	·46	·50	·49	·57	—
14	38	20	·01	·26	·44	·48	·50	·68	·54	·16	—	·49	1·4	4·0	5·9	8·5	5·7	·88
02	40	20	·02	·16	·23	·26	·25	·22	·14	·04	—	·01	·40	·60	·60	·70	·32	·01
25	40	20	·03	·26	·18	·16	·15	·09	·08	·02	—	·62	·66	·22	·18	·07	—	—
56	46	20	·02	·03	·05	·05	·07	·04	·03	·01	—	—	No haemoglobinuria					
56	46	30	·03	·06	·05	·19	·50	·37	·24	·06	—	—	—	·07	·30	·98	·70	—

Discussion

Haemoglobinuria was observed in sheep during the intravenous injection of an aqueous solution of 20% propylene glycol administered at 10 min. intervals for 2 hr. Proprietary preparations of pentobarbitone sodium, which contain propylene glycol in similar proportions, caused haemoglobinuria when used to produce anaesthesia in sheep.

The haemoglobinuria is invariably accompanied by intravascular haemolysis. There was considerable individual variation in the degree of haemolysis and haemoglobinuria, and from Table I it may be seen that, although haemoglobinuria was produced in most sheep by 20% propylene glycol in water, the concentration of the glycol had to be increased to 30% to cause haemoglobinuria in sheep No. 56. The difference in individual response is also seen in Fig. 1, which shows that the haemoglobinaemia in sheep No. 04 was relatively great, yet the amount of haemoglobin measured in the urine was less than that of the sheep in Table I. The low concentration of haemoglobin present in the urine was not due to diuresis because the volume of urine passed by sheep No. 04 during the experimental period did not exceed that passed by the other sheep. Tubular reabsorption of haemoglobin from the kidneys might also be discounted as haemosiderin was not found in the urine up to 24 hr. after haemoglobin appeared there.

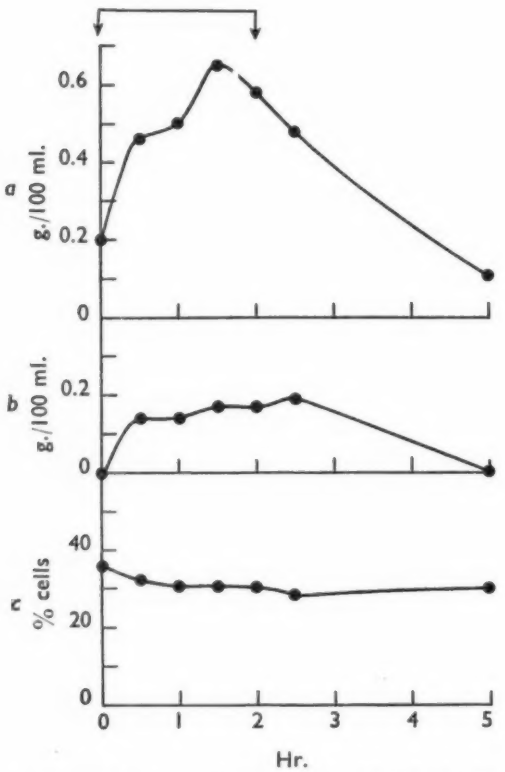

Fig. 1.—Haemoglobin concentration in plasma (*a*) and urine (*b*) and haematocrit (*c*) of sheep No. 04. 15 ml. of 20% propylene glycol was injected at first arrow, 2 ml./10 min. until second arrow.

Lehman and Newman (1937), in a report on the haemolytic action of a 33.3% solution of propylene glycol dissolved in normal saline and injected into the blood stream of dogs, rabbits and chickens, suggested that the blood cells are destroyed osmotically in passing through the glycol at the site of the injection. If this were the case in the sheep, the first sample of plasma taken after the initial injection of 15 ml. would be expected to contain more haemoglobin than the subsequent samples which were taken when only 2 ml. of the glycol was being injected at 10 min. intervals. This was not so, and there was no haemoglobinuria after intravenous injections of similar quantities of distilled water and 20% glycerol in water. These facts indicate that propylene glycol had some action on the blood cells other than a simple osmotic effect.

The anomalous *in vitro* effects of mixing whole blood with 20% propylene glycol in water seem to indicate that the red cells of individual sheep vary in their sensitivity to propylene glycol, and this may explain the variation in extent of haemolysis that was observed after propylene glycol had been injected.

It was interesting to observe that when physiological saline was substituted for water in the preparation of the 20% propylene glycol haemoglobinuria did not occur. However, when the sheep received an aqueous injection of 20% propylene glycol 5 min. after an initial injection of 100 to 150 ml. of physiological saline, haemoglobinuria was sometimes observed, particularly if a transient diuresis followed the saline injection. In view of the evidence cited against osmotic effects, this suggests that saline may exert some sort of protective action against the haemolytic effects of propylene glycol.

It was also interesting that haemoglobin was not found in the urine of rabbits, unlike that of sheep, when the animals were anaesthetized with an equivalent amount of a solution of pentobarbitone sodium containing propylene glycol.

It thus appears that the sheep may be particularly susceptible to the haemolytic action of propylene glycol, and the incorporation of the latter in pharmaceutical preparations for intravenous administration to sheep would appear to be unwise.

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THE RELEASE OF ADRENALINE AND NORADRENALINE FROM THE ADRENAL MEDULLA OF THE CAT DURING SPLANCHNIC STIMULATION

BY

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Cats were anaesthetized with chloralose, eviscerated and the right adrenal gland was removed. The venous outflow from the left adrenal gland was collected during 10 five-minute periods of stimulation of the left splanchnic nerve. The amounts of adrenaline and noradrenaline in the venous outflow and in the stimulated and unstimulated glands were determined by a fluorimetric method. In eight experiments a mean of 50.5 μg. of total catechol amine was recovered from the effluent blood. The mean difference in amine content between the stimulated and unstimulated glands was 50 μg., representing a loss of 29% from the stimulated gland. The proportions of the two amines in the effluent blood were very similar to those found in the gland. The results provide no evidence for an increase in the rate of synthesis of catechol amines during splanchnic stimulation.

In 1912 Elliott reported a "very slight" or "almost inappreciable" loss of adrenaline from the stimulated adrenal glands of cats in which he stimulated the splanchnic nerve for periods of 2 to 7 hr. Elliott (1912) had expected to find a greater degree of depletion since the blood pressure responses indicated that pressor amine had been discharged from the gland during each period of stimulation. Tscheboksaroff (1911) had made similar experiments in dogs and reported that the stimulated gland contained more adrenaline at the end of the experiment than the unstimulated gland. West (1950) obtained similar results in rabbits. A possible explanation of these findings is that the rate of synthesis had kept pace with the rate of release of amine so that depletion did not occur. Recently Holland and Schümann (1956) provided further evidence in support of this theory. In a series of experiments in eviscerated cats they showed that the adrenal gland lost 25% of its amine content when stimulated through the splanchnic nerve, but the amount of amine estimated to have passed into the circulation was more than 63% of that originally present in the gland. They concluded that the difference (38%) must have been synthesized during splanchnic stimulation. This evidence implies a rapid rate of amine synthesis during stimulation.

Such an interpretation is not in agreement with other information available concerning the rate of synthesis of medullary amines. Insulin hypoglycaemia causes depletion of adrenal medullary amines, and recovery of the normal amine content of the gland is not complete for several hours or days in cats (Burn, Hutcheon, and Parker, 1950) or in rats (Hökfelt, 1951; Outschoorn, 1952). Outschoorn (1952) also found that, after depletion with β-tetrahydronaphthylamine, recovery of the initial amine concentration in the gland took about 16 hr. A similar slow replacement of discharged amines has been demonstrated by Van Arman (1950) using eserine in rats. Further, Butterworth and Mann (1957a) found that restoration of the normal total amine content of the cat adrenal glands required 7 days following depletion by repeated doses of acetylcholine. The delayed recovery of normal amine content after insulin hypoglycaemia, β-tetrahydronaphthylamine or eserine, which act by way of the splanchnic nerves, or after acetylcholine, indicates a slow rate of synthesis of medullary amine for which more evidence has recently been obtained by Butterworth and Mann (1957b). They found that the adrenaline and noradrenaline lost from the cat adrenal gland, stimulated by repeated doses of acetylcholine, could be recovered quantitatively in the adrenal blood.

It is difficult to reconcile these findings with the suggestion that rapid synthesis occurs during electrical stimulation of the splanchnic nerves. For this reason we decided to reinvestigate the problem. We estimated directly the amount of catechol amines appearing in the adrenal venous effluent during electrical stimulation of the splanchnic nerve and compared this with the difference in amine content of the stimulated and unstimulated glands.

METHODS

Cats of either sex, weighing 2 to 4 kg., were anaesthetized with ether followed by chloralose, 60 mg./kg. intravenously. The stomach, intestines, spleen and pancreas were removed; the blood supply to the liver was interrupted by a ligature round the coeliac artery. In three experiments the renal vessels were also tied off. The blood pressure was recorded from a carotid artery. The right adrenal gland was removed and immediately weighed, ground in 0.5 ml. of 0.15 N-HCl and frozen. Heparin (116 i.u./kg.) was injected intravenously. Artificial respiration was begun and the left splanchnic nerve was freed by dissection under the diaphragm and cut as high up as possible. The lesser splanchnic nerves and the sympathetic chain on the same side were also cut.

For collection of the adrenal venous outflow, the usual procedure was to insert a polyethylene cannula into the adreno-lumbar vein just lateral to the adrenal gland. When the adrenal vein was tied close to the inferior vena cava, blood from the gland flowed into the cannula. This blood could then be collected, or returned to the animal by a second polyethylene tube in the right renal or other convenient vein.

The splanchnic nerve was stimulated with rectangular pulses at a rate of 20/sec. (duration 2.5 msec., intensity 5 V.). The venous effluent from the gland was collected during 10 periods of stimulation. Each collection was continued for 15 sec. longer than the period of stimulation (4 min. 45 sec.) to complete the recovery of released amine. There was a rest period of 7 min. between each collection. The volume of blood collected in 5 min. varied from 3.4 to 11.0 ml. in different cats. To maintain the blood volume, arterial blood from another cat (anaesthetized with ether and chloralose and given heparin) was injected after every second collection of adrenal venous blood; the volume injected was equal to that lost by the experimental animal. Control samples of adrenal venous blood were collected without stimulation of the splanchnic nerve.

At the end of the experiment the left gland was removed, weighed, ground in 0.5 ml. of 0.15 N-HCl and frozen.

Standard Solutions.—Double-distilled water was used for the preparation of solutions and of samples for assay. Stock solutions of adrenaline hydrogentartrate and noradrenaline hydrogentartrate monohydrate containing 1 mg./ml. free base were prepared in 0.1 N-HCl and diluted as required to provide standard solutions. Values for adrenaline and noradrenaline were calculated as the free base.

The Assay of Adrenaline and Noradrenaline in Plasma.—The venous effluent was collected in cold, graduated centrifuge tubes containing 12 i.u. of heparin and immediately centrifuged for 5 min. at 600 *g*. The "stimulated" plasma was collected and pooled as required, and either frozen or kept at 2° for 12 to 14 hr. before purification.

1.5 to 3 ml. of plasma was diluted to 10 times the original volume with Versene buffer (0.7% Versene in 1 M-sodium acetate solution, adjusted to *p*H 8.5 with NaOH). The diluted plasma was passed down columns (0.6 cm. internal diameter) containing 2 g. of acid-washed aluminium oxide (Merck—reagent grade) which had been washed in the Versene buffer solution; the rate of flow was 36 drops/min. Following adsorption, the column was washed with 5 ml. of Versene buffer and 5 ml. of water. The amines were eluted from the column at a flow rate of 9 drops/min. using 5 ml. of 0.2 N-acetic acid followed by 5 ml. of water.

Duplicate determinations of the adrenaline and noradrenaline content of the purified plasma were made using a differential fluorescence method similar to that described by Millar and Benfey (1958). The adrenaline and noradrenaline were oxidized by potassium ferricyanide at *p*H 6.0 and the fluorescence intensity, developed after treatment with sodium hydroxide and ascorbic acid, was measured at 365 mμ and 436 mμ using a Beckman D.U. fluorescence attachment. Calculation of the amount of each amine present in the eluate was made using a simultaneous equation derived from the fluorescence shown by the "stimulated" plasma minus that shown by the control plasma and the fluorescence values of standard solutions containing 0.1 μg. of adrenaline or noradrenaline.

The Assay of Adrenaline and Noradrenaline in Adrenal Glands.—The amines were separated by paper chromatography and estimated separately by fluorimetry after elution from the paper. The homogenates of the stimulated and non-stimulated glands were thawed and transferred with washing (0.01 N-HCl) to centrifuge tubes and centrifuged at 11,000 *g* for 10 min. at 0°. The supernatant fluid was carefully removed to avoid, as far as possible, contamination with the floating lipid layer. The sediment was twice washed with 0.3 ml. of 0.01 N-HCl and the washings were added to the supernatant. The final volume of the gland extract varied between 2.2 ml. (stimulated) and 3.0 ml. (unstimulated) so that the final concentration of amine was similar in each extract. For chromatography, 0.07 ml. of extract was applied, along a 4 cm. origin, to Whatman No. 1 filter paper which had been previously washed by three successive immersions for 12 hr. in 0.01 N-HCl. Ascending chromatography in the dark for 15 hr. at 26° in phenol-HCl (Vogt, 1952) was used for separation of the amines. The chromatograms were

washed in reagent grade benzene to remove excess phenol. For each extract, a portion of the same extract to which 3 μg. of adrenaline and 3 μg. of noradrenaline had been added was run as a control. The control strip was sprayed with alkaline potassium ferricyanide to develop the adrenaline and noradrenaline spots, which were used as a guide for cutting out 5 × 7 cm. areas of filter paper for elution. These sections were cut into small pieces and extracted for 8 hr., with occasional shaking, in 0.02 N-acetic acid. The extracts were assayed in duplicate for adrenaline and noradrenaline by measuring the fluorescence at 365 mμ. Standard curves for each amine (0.1 to 0.3 μg.) were prepared for each determination.

The Recovery of Adrenaline and Noradrenaline from Whole Blood.—In a separate series of experiments, blood from the inferior vena cava of cats anaesthetized with chloralose and injected with heparin was separated into 5 ml. portions and chilled in an ice bath. A total volume of 0.65 ml. of 0.001 N-HCl in 0.9% NaCl solution, containing both adrenaline and noradrenaline (0.3 to 2.0 μg. of either amine), was added to each sample except the control, to which acid saline only was added. The samples were mixed and centrifuged for 5 min. at 600 *g* and the plasma was collected. The adrenaline and noradrenaline present in the plasma were determined as previously described. In 10 tests, the mean recovery of adrenaline was 77.8% ±3.4 (S.E.) and of noradrenaline 70.35% ±2.8 (S.E.). As there was no significant difference between the two means, they were averaged to give a mean recovery value of 74.1%. The experimental values for plasma were suitably corrected to give values for whole blood.

Results

A summary of the results obtained in eight experiments is shown in Table I. An average of 50 μg. of catechol amine was released from the adrenal gland during 10 periods of stimulation (lasting a total of 50 min.) as determined by the difference in amine content between the right, non-stimulated, gland and the left, stimulated, gland (171 − 121 = 50 μg.). This value represents a mean release of catechol amines during splanchnic stimulation of 29%. The catechol amine present in the venous blood collected from the adrenal gland during splanchnic stimulation ranged from 13 to 107 μg., with a mean of 51.5 μg. The stimulated gland always contained less catechol amine at the end of the experiment than did the non-stimulated gland which was removed at the beginning. In each experiment, the amount of amine present in the venous outflow was similar to that estimated to have been lost from the stimulated gland. Similar results have been obtained in three other experiments in which the frequency of stimulation was varied and the plasma samples were assayed individually without pooling.

In Table II, further results from the same eight experiments are presented. The values shown are for adrenaline as % total catechol amine (adrenaline + noradrenaline) in the adrenal glands and in the pooled plasma samples. There does not appear to be any important alteration in the proportion of adrenaline and noradrenaline in the stimulated gland or in the effluent blood, irrespective of the degree of depletion observed or the initial amount of noradrenaline present in the gland. The mean value for adrenaline in the non-stimulated gland was 62% and that in the stimulated gland 64%. The greatest difference found was a 5% increase in the adrenaline of the stimulated gland of cat No. 7. There appears to be a

Table I

THE RELEASE OF CATECHOL AMINES FROM THE ADRENAL MEDULLA DURING SPLANCHNIC NERVE STIMULATION

Left gland stimulated, right not stimulated. Amine content of blood adjusted for loss in extraction: see text.

Cat No.	Total Amine Content (μg./Gland)		Amine Released (μg.)	Amine in Blood (μg.)	% Amine Released
	Right	Left			
1	134	94	40	47	30
2	232	197	35	24	15
3	168	111	57	59	34
4	208	157	51	54	24·5
5	87	67	20	13	23
6	135	101	34	35	25
7	218	144	74	73	34
8	185	95	90	107	49
Mean	171	121	50	51·5	29

Table II

ADRENALINE AS % OF TOTAL CATECHOL AMINE CONTENT OF ADRENAL GLANDS AND OF POOLED PLASMA SAMPLES

Left gland stimulated, right not stimulated. No. of plasma samples pooled for analysis shown in brackets. Cat No. 1 excluded from means.

Cat No.	% Adrenaline in Gland		% Adrenaline in Plasma (Pooled)		
	Right	Left	1 (5)	2 (5)	3
1	57	62	46	43	
			(3)	(3)	(4)
2	72	75	63	67	68·5
3	67	67	57	58·5	67·5
4	67	69·5	44	51	56
5	61	62	57·5	59	53
6	37	36	43	44	48
7	55	60	47	56·5	58
8	73	77	54	60	74
Mean (2–8)	62	64	52	58	61

smaller proportion of adrenaline in the first pooled plasma samples than in the final pooled samples. The mean value for adrenaline in the first pooled sample of plasma was 52%, compared with 61% in the last sample.

Discussion

The results provide no evidence for an increase in the rate of formation of catechol amines during electrical stimulation of the splanchnic nerve. This finding differs from that of Holland and Schümann (1956). They reported a mean depletion of the stimulated gland of 25%, which is very similar to the 29% depletion we observed under similar experimental conditions. However, we were unable to confirm their finding that synthesis occurred to the extent of 38% of the amine content of the unstimulated gland (mean of twelve experiments). The reason for this difference is not known, but two possibilities suggest themselves. First, the assay method used by these workers may not be suitable for estimating the output of medullary amines in the same animal. It is open to question whether the infusion of a 50:50 mixture of adrenaline and noradrenaline at a constant rate into the external jugular vein is capable of duplicating quantitatively the effect of amine released into the circulation by stimulation of the splanchnic nerve. Second, the possibility cannot be overlooked that part of the pressor response which these authors observe after splanchnic stimulation is due to the release of pressor substances from areas other than the adrenal gland. In five of our eight experiments no pressor effect was observed during the periods of splanchnic stimulation with collection of the blood from the adrenal gland. In one cat in which the branches of the coeliac artery were ligated as close as possible to the organs which they supplied, a pressor response was observed. Following the application of a second ligature to the coeliac artery close to its origin from the aorta, there was no pressor response to any of the subsequent periods of splanchnic stimulation. In the other two cats each period of splanchnic stimulation was accompanied by some rise in blood pressure. After removal of the stimulated gland in these cats, splanchnic stimulation still caused a rise in blood pressure in the now bilaterally adrenalectomized animal. These observations make it unlikely that the pressor response was due to the escape of amine from the stimulated gland but do support the possibility that pressor substances may arise from elsewhere than the adrenal gland.

It is clear from previous work (Elliott, 1912; Crowden, 1929; Van Arman, 1950) that synthesis of amine by the adrenal medulla is not necessarily dependent upon the integrity of the sympathetic nerve supply. These workers found that the amine content of a depleted adrenal gland could return to normal despite cutting of the ipsilateral splanchnic nerve. As pointed out earlier, it is difficult to reconcile the rapid synthesis of amine postulated by Holland and Schümann (1956) with the fact that, after insulin and other agents which eventually act through splanchnic nerves to deplete the gland, recovery of the normal content of amine requires several hours or days. Tscheboksaroff (1911) found that in the dog anaesthetized with morphine and curarized, stimulation of the cut left splanchnic nerve led to release of pressor substance; at the end of the experiment the pressor activity of an extract of the stimulated gland was generally greater than that of a similar extract from the right unstimulated gland. He concluded that adrenaline "wird auch in grösserer Menge gebildet und im Drüsenparenchym selbst angesammelt." However, the splanchnic nerve supply to the unstimulated gland apparently remained intact throughout the experiment and the gland was therefore subject to depletion caused by the central action of morphine. The apparent increase in activity in the left gland might equally well represent a greater depletion of the right gland. It is therefore difficult to accept these experiments as evidence for an increase in the rate of synthesis in the stimulated gland. Elliott (1912) never succeeded in demonstrating more than "a very slight loss" of medullary amines when using the method of measuring the residual adrenaline in the gland after stimulation. However, in the one cat experiment (out of 10) which he quotes in detail, there was a 34% depletion of the gland which had been stimulated intermittently for 7 hr. In the three dog experiments the stimulated gland showed a 3% loss in one, a gain of 7% in another, and a loss of 13% in the third. These experiments, like those of Tscheboksaroff (1911), do not necessarily support the idea that splanchnic stimulation causes a rapid rate of synthesis of catechol amine. The results reported by West (1950) show that the stimulated adrenal gland of the rabbit contained an average of 36% more adrenaline than the unstimulated gland. Like Tscheboksaroff (1911), he apparently did not cut the right splanchnic nerve and used an anaesthetic (urethane) which Elliott (1912) had shown to cause a depletion of the adrenal medulla. From these observations it is difficult to conclude

that an increase in the rate of synthesis of catechol amine with stimulation of the splanchnic nerve has been adequately demonstrated.

In six of our eight experiments we observed a small increase (1 to 5%) in the % of adrenaline in the stimulated compared with the non-stimulated gland. These small changes may be insignificant. If they do have any meaning, two explanations should be considered. The first is that they represent, as Holland and Schümann (1956) concluded, an increase in the rate of methylation of noradrenaline. The second explanation, that more noradrenaline than adrenaline was released from the store in the gland, is consistent with our finding that the earlier samples of plasma do contain a higher proportion of noradrenaline than the stimulated gland. The proportion of noradrenaline in later samples of plasma approximates more closely to that in the glands.

While the rate of new formation of amines by the gland may be fast enough to keep pace with the resting secretion it does not appear to be sufficient to meet the demand of any severe or prolonged stimulus; under these conditions most of the amine liberated will be drawn from the storage depots, thus depleting them.

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PROTECTION AGAINST SYSTEMIC POISONING BY MUSTARD GAS, DI(2-CHLOROETHYL) SULPHIDE, BY SODIUM THIOSULPHATE AND THIOCIT IN THE ALBINO RAT

BY

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The lethal effects of mustard gas, di(2-chloroethyl) sulphide, in the albino rat have been counteracted by Thiocit, a mixture of sodium thiosulphate and trisodium citrate in the ratio 10 : 1, administered intraperitoneally in a dose of 2.75 g./kg. Thiocit afforded complete protection against greater than the median lethal dose of mustard gas whether given 10 min. before or 10 min. after mustard gas and raised the LD50 of mustard gas by approximately three times. The protection appeared whether the total dose of Thiocit was given in one injection or serially over 30 min. The effective doses of sodium thiosulphate and of Thiocit in rats were of the order of 3.0 g./kg. Sodium thiosulphate alone and Thiocit have been administered in single doses by slow infusion, by stomach tube and in drinking water. Both have shown activity by all routes of administration, but activity was greatest by intraperitoneal injection. The use of Thiocit in conjunction with mustard gas therapy is suggested.

Many attempts have been made to find a substance which offers effective protection against the systemic effects and death produced by poisoning with mustard gas, di(2-chloroethyl) sulphide. Numerous authors have demonstrated some alleviation of the local or systemic effects of mustard gas by cysteine and glutathione. Paulet and Chappet (1956) have discussed the more recent work and showed that phenylallylthiourea was as active as cysteine, but somewhat more effective than glutathione in reducing the severity of the leucopenia produced by sublethal injections of mustard gas into albino rats.

In spite of this extensive search, however, little success has been achieved in protecting against lethal doses of mustard gas. A large number of thio compounds has been tested on the basis of theoretical bonding with, and high competition factor for, mustard gas (Ogston, 1948). Sodium thiosulphate has a high competition factor (2.7×10^4), and has probably a relatively high permeability through the cell membrane ; it is non-toxic in very high doses and theoretically could combine with mustard gas. Citrates also have a high competition factor (1.7×10^2) (Callaway and Pearce, unpublished observations) for mustard gas ; they are natural constituents of body fluids and probably possess a fairly high permeability at the cellular level.

These two compounds were therefore investigated for possible prophylactic or therapeutic effects against poisoning by mustard gas. Each was tried separately and also in combination as Thiocit by several routes against lethal doses of the mustard gas.

METHODS

Sodium thiosulphate 500 mg./ml. and sodium citrate 100 mg./ml. were used in aqueous solution both for injection and for drinking purposes. Thiocit consisted of $Na_2S_2O_3,5H_2O$ (50 g.) and $Na_3C_6H_5O_7,5H_2O$ (5 g.) made up to 100 ml. with distilled water to give a total concentration of 550 mg./ml.

Male albino rats of the Wistar strain weighing 190 to 215 g. were used. Mustard gas solution in propylene glycol was freshly prepared for each experiment, such that the volume injected in ml. was equal to the body weight in kg. The agents were usually administered either 10 min. before (−10′) or 10 min. after (+10′) mustard gas.

After injection, the rats were returned to their cages and their general condition noted and survival times observed. No attempt was made to observe the blood picture, since the main objective was to find the best combination of time of injection and dose for protection against death from mustard gas poisoning.

Mustard gas was always administered subcutaneously into the loose skin of the right groin, and materials used in prophylaxis or therapy were

administered subcutaneously, intraperitoneally, intravenously using the lateral marginal vein (Pearce, 1957), or orally, as indicated in the results.

The Determination of Competition Factor. —The rate of liberation of acid from mustard gas in a solution of a non-acid-forming competitor under investigation was determined in the presence of a known concentration of a suitable acid-forming competitor of known competition factor in the manner described by Ogston (1948). The competition can then be calculated from the expression given by Ogston (1948), namely, molar concentration × competition factor.

Results

Control Injections of Mustard Gas

The LD50 of mustard gas when administered subcutaneously to rats was 3.8 mg./kg. From the dosage/mortality curve the LD99 was predicted to be 6.27 mg./kg., and, in fact, no survivors occurred at this dose, almost all the rats dying between the third and fifth day. The animals showed severe emaciation, increasing leucopenia (Callaway and Pearce, unpublished observations), diarrhoea, loss of weight and marked deterioration in the condition of the fur. On the third or fourth day they usually became progressively weaker and died.

Sodium Thiosulphate by Intraperitoneal Injection.—These results are summarized in Table I. The best protection was obtained from a single intraperitoneal injection of sodium thiosulphate (2.75 to 3 g./kg.) given 10 min. before 6.27 mg./kg. mustard gas. Protection also occurred when the dose of mustard gas was increased to 8.15 mg./kg. and only 2/10 animals died. In nearly all instances the survivors remained actively healthy, though some were in poor condition for a day or two.

TABLE I

THE EFFECT OF INJECTING SODIUM THIOSULPHATE INTRAPERITONEALLY INTO MALE ALBINO RATS 10 MIN. BEFORE THE SUBCUTANEOUS INJECTION OF MUSTARD GAS

Treatment with sodium thiosulphate was given 10 min. before mustard gas injection.

Dose of $Na_2S_2O_3,5H_2O$ (g./kg.)	Dose of Mustard Gas (mg./kg.)	No. of Deaths: Day of Death							Total Mortality
		1	2	3	4	5	6	11	
	6·27				5				5/5
	3·8				2				2/5
3·0	6·27				1				1/5
3·0	3·8								0/5
	6·27				10				10/10
3·0	6·27				1				1/10
	6·27				9	1			10/10
2·75	6·27				1				1/10
	6·27			1	4				5/5
2·75	6·27							1	1/5
1·5	6·27							1	1/5
	10·6			1	4				5/5
2·75	10·6			2	5	1	2		10/10
2·75	8·15				2				2/10
2·75	6·27				1				1/10
	6·27				9				10/10

Sodium Thiosulphate by Intraperitoneal and Subcutaneous Infusion.—To decide whether prolonged administration would be as effective as a massive initial dose in giving protection, peritoneal and subcutaneous infusion experiments were undertaken. Sodium thiosulphate solution was injected gradually over a period of 33 min. immediately following the subcutaneous injection of mustard gas until the total amount received by each rat was 3 g./kg. in a total volume of about 1.2 ml. A control group received 0.85% sodium chloride solution instead of sodium thiosulphate under exactly the same conditions. In the group treated intraperitoneally, the mortality was 4/5, whilst in the group treated subcutaneously and in the control group the mortality was 5/5.

Oral Administration of Sodium Thiosulphate

In Drinking Water.—Previous work has indicated that oral therapy would be unlikely to be successful, but, in view of the success with intraperitoneal injections, 20 rats were divided into 4 groups of 5, which were treated in the following manner.

Group A was given normal drinking water daily for 7 days, then injected with a dose of mustard gas subcutaneously (4.0 mg./kg., being very slightly above the LD50), and continued with their normal drinking water. The mortality was 4/5.

Group B were offered a 0.5% aqueous solution of sodium thiosulphate in place of their normal drinking water daily for 7 days, then injected with a dose of mustard gas subcutaneously (4 mg./kg.) and continued drinking 0.5% thiosulphate for a further 7 days. The mortality was 2/5.

Group C were treated like those in Group B except that they received no mustard gas injections. The mortality was 0/5.

Group D received normal drinking water for 7 days, then were injected with a dose of mustard gas (4 mg./kg.) and given 0.5% sodium thiosulphate solution instead of drinking water for 7 days. The mortality was 1/5.

The drinking fluid was replaced daily and the intake observed, there being no difference between test and control. The weather was warm and approximately 30 to 50 ml. was drunk by each rat daily. The results suggested that there may have been a small measure of protection, though this was not statistically significant ($P>0.1$).

TABLE II

THE EFFECT OF GIVING SODIUM THIOSULPHATE BY STOMACH TUBE ON THE TOXICITY OF MUSTARD GAS TO RATS

Sodium thiosulphate was given 10 min. before (−10′); at (0′); 10, 20 or 30 min. after (+10′, +20′ or +30′) the mustard gas. In Expt. 5, sodium thiosulphate was given dissolved in 25% ethanol to find whether absorption was more rapid.

Expt. No.	No. of Animals	$Na_2S_2O_3,5H_2O$ (g./kg.)		Mustard Gas (mg./kg.)	$Na_2S_2O_3,5H_2O$ (g./kg.)			Final Mortality	Remarks
		−10′	0′	0′	+10′	+20′	+30′		Condition of Rats on Days Following Treatment (for example +3=3rd day after treatment)
1	10	2·0	2·0	6·27	2 0			5/10	9/10 poor on +3, 5/10 recovered. Deaths +4, +5
	10	—	—	6·27	—			10/10	Poor +3, dead +4
2	10	2·0	1·0	6·27	1·0			6/10	On +3 4 poor, 5 good, 1 dead. Deaths +3, +4
	10	—	—	6·27	—			10/10	Poor +3, dead +4
3	5	5·0		6·27				5/5	
	5		5 0	6·27				5/5	
	5			6·27	5 0			4/5	Nearly all poor +3, had diarrhoea, dead +4
	5			6·27		5 0		5/5	
	5			6·27			5·0	4/5	
	5			—			5.0	0/5	All quite fit throughout
	5			6·27				5/5	All poor, diarrhoea +3, dead +3, +4
4	5	1·5	1·5	6·27	1·5			4/5	+14 day terminated. 1 fit
	5	1·0	1·0	6·27	1·0			5/5	
	5		1·0	6·27	1·0	1·0		4/5	1 fit
	5			6·27	1·0	1·0	1·0	4/5	1 fit
	5			—	1·0	1·0	1·0	0/5	5 fit
	5			6·27				5/5	
5	5			6·27	0·6	0·6	0·6	3/5	2 fit
	5			6·27	0·9	0·9	0·9	5/5	
	5			6·27	1·2	1·2	1·2	3/5	2 fit
	5			6·27	1·5	1·5	1·5	4/5	1 fit
	5			—	1·5	1·5	1·5	0/5	5 fit
	5			6·27				5/5	

By Stomach Tube.—The results of administering sodium thiosulphate by stomach tube are given in Table II. The protection against the LD99 dose of mustard gas appeared to be better if the total dose was given in several instalments, close to the time of the mustard gas injection. Although nearly 50% survival was obtained against an LD99 dose of mustard gas, the animals were sometimes in poor condition on the third day but recovered later.

Sodium Thiosulphate by Intravenous Injection

A certain amount of protection against the LD99 of mustard gas was shown when 1.5 g./kg. sodium thiosulphate was injected intravenously at the same time as the subcutaneous injection of mustard gas (1/5 died) or 10 min. afterwards (3/5 died). All the control rats died (5/5).

Thiocit by Intraperitoneal Injection

The preceding experiments had shown that a reasonable protection against the lethal effects of mustard gas could be achieved with sodium thiosulphate, though in no instance was it complete. It seemed likely that the addition of a second competing ion, namely citrate administered simultaneously with the thiosulphate, might produce an even better protection. As we found that sodium citrate alone showed no promise against mustard gas poisoning, a solution of Thiocit was prepared as previously described. Table III gives the results of 5 separate experiments using Thiocit injected intraperitoneally 10 min. before or after the injection of mustard gas subcutaneously. All the controls died (25/25) after receiving 6.27 mg./kg. mustard gas (LD99) whereas none (0/25) died among those treated with 2.75 g./kg. of Thiocit. When the dose of Thiocit was reduced to 1.65 g./kg., 1 out of 5 died at the LD99 of mustard gas. Effective protection occurred even when the dose of mustard gas was increased to 8.15 mg./kg. and 10.6 mg./kg., the mortality being only 2 out of 10 at each dose, and the survivors remained in good condition, whereas 8 out of 10 died with 13.8 mg./kg. of mustard gas.

The results of injecting Thiocit (2.75 g./kg.) 10 min. after mustard gas (6.27 mg./kg.) showed that a completely successful treatment could be achieved against the lethal effects of this dose of mustard gas (Table III, Expt. 5). All the treated rats survived (0/10 died) compared with complete mortality among the controls (10/10 died). The survivors were in good condition and have been kept for 6 months during which time they have shown good general progress and health.

TABLE III

THE EFFECTS OF THIOCIT INJECTED INTRAPERITONEALLY BEFORE OR AFTER THE SUBCUTANEOUS INJECTION OF MUSTARD GAS IN RATS

Thiocit was injected 10 min. before (in Expts. No. 1 to 4) and 10 min. after (in Expt. No. 5) mustard gas. All the survivors were healthy 6 months later.

Expt. No.	Thiocit (g./kg.)	Mustard Gas (mg./kg.)	No. of Deaths: Day of Death 1	2	3	4	5	6	7	8	Total Mortality
1	2·75	Control									0/5
	2·75	6·27									0/5
	1·65	6·27				1					1/5
		6·27			1	4					5/5
2	2·75	6·27									0/10
		6·27			3	6		1			10/10
3		10·6			1	4					5/5
	2·75	10·6			1		1				2/10
	2·75	8·15				1	1				2/10
	2·75	6·27									0/10
		6·27				9	1				10/10
4	2·75	17·9			1	8	1				10/10
		13·8			5	5					10/10
	2·75	13·8			1	6	1				8/10
5		6·27				8	2				10/10
	2·75	6·27									0/10

Thiocit by Mouth

A dose of 3.3 g./kg. of Thiocit was administered by stomach tube at various times before and after 6.27 mg./kg. of mustard gas. Some protection was shown (Table IV), but this was not very marked, the total combined mortality in the treated groups being 15/20 compared with 5/5 in the untreated controls.

TABLE IV

THE EFFECT OF ADMINISTERING THIOCIT ORALLY ON THE TOXICITY OF MUSTARD GAS TO RATS

For explanation of −10′, +10′ and +20′, see Table II.

Thiocit (g./kg.) −10′		Mustard Gas (mg./kg.)	Thiocit (g./kg.) +10′	+20′	No. of Deaths: Day of Death 1	2	3	4	5	6	12	Total Mortality
3·30		6·27						1	1	1	1	0/5
3·30	3·30	6·27						4				4/5
		6·27	3·30				1	1				2/5
		6·27		3·30			2	3				5/5
		6·27					3	1	1			5/5

DISCUSSION

Our results showed that sodium thiosulphate alone, but more particularly when combined with sodium citrate as Thiocit, was capable of protecting rats completely from a dose of mustard gas which would otherwise kill 99% of rats. Although a certain amount of protection occurred following the oral administration of both preparations, the most effective route was intraperitoneal. The results from four experiments in which 2.75 g./kg. of Thiocit was injected intraperitoneally either 10 min. before or 10 min. after 6.27 mg./kg. of mustard gas showed no deaths (0/35) among the treated rats compared with 35/35 deaths among the controls. In Table V, the mortality resulting from thiosulphate and Thiocit injected either 10 min. before or 10 min. after various doses of mustard gas is compared. It will be seen that the LD50 of mustard gas when Thiocit was used was raised approximately 3 times.

TABLE V

THE SUMMARIZED MORTALITIES FOLLOWING THE INTRAPERITONEAL INJECTION OF THIOSULPHATE OR THIOCIT WITH VARIOUS DOSES OF MUSTARD GAS

Dose of Mustard Gas (mg./kg.)	Thiosulphate (2·75 g./kg.)	Thiocit (2·75 g./kg.)	Mustard Gas Alone
17·90	—	10/10	10/10
13·78	—	8/10	10/10
10·60	10/10	2/10	5/5
8·15	2/10	2/10	—
6·27	1/10	0/10	10/10

Holiday, Philpot and Stocken (1950) have shown that sodium monothiophosphate is a weak antidote to mustard gas. They treated rats with 5 mg./kg. of mustard gas dissolved in ethanol. The 10-day mortality was reduced from 10/20 to 2/10 by sodium monothiophosphate.

Of the many substances which we have tested against the systemic effects and death from mustard gas poisoning, only hydroxylamine (Callaway and Pearce, unpublished observations) apart from Thiocit had hitherto shown any promise. Hydroxylamine, however, was unstable and caused occasional deaths from pneumonia. It was only effective under restricted conditions of *p*H, and then only for a short period in relation to the time of mustard gas injection, and was therefore not investigated further.

This work has shown that sodium thiosulphate and Thiocit are effective antidotes to mustard gas. In view of the potential value of mustard gas and its analogues in certain clinical conditions, the existence of the means of counteracting their effects may be of value. Thiocit may also provide a means of elucidating the mechanism by which systemic damage and death is caused in poisoning by mustard gas.

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THE EFFECT OF SOME OXIMES IN SARIN POISONING

BY

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The effects of mono*iso*nitrosoacetone (MINA), diacetylmonoxime (DAM) and pyridine-2-aldoxime methiodide (P2AM) upon the cholinesterase of sarin poisoned rats have been studied. Mono*iso*nitrosoacetone and diacetylmonoxime given before sarin protected blood and brain cholinesterase from inhibition. Mono*iso*nitrosoacetone given after the appearance of signs of poisoning caused a rapid reactivation of brain cholinesterase. Diacetylmonoxime, at an equimolar dose, produced only a slight increase in enzyme activity, and pyridine-2-aldoxime methiodide, the best reactivator *in vitro*, reactivated blood but not brain cholinesterase. There is a relationship between protection and reactivation of brain cholinesterase and prevention and alleviation of signs of poisoning.

Several oximes, which are known to react with *iso*propyl methylphosphonofluoridate (sarin) *in vitro* and to reactivate cholinesterase inhibited by this substance, have recently been found to be effective against sarin poisoning in animals (Green and Saville, 1956; Childs, Davies, Green, and Rutland, 1955; Askew, 1956, 1957). No clear relationship between their *in vitro* activity and *in vivo* potency is obvious, however. Thus the best reactivator, pyridine-2-aldoxime methiodide, is the weakest antidote and mono*iso*nitrosoacetone, which is a faster reactor with alkylphosphates and a more rapid reactivator of the inhibited enzyme than diacetylmonoxime, is no better therapeutically than the latter.

In order to examine this discrepancy more closely, the effect of mono*iso*nitrosoacetone, diacetylmonoxime, and pyridine-2-aldoxime methiodide has been studied in rats in sarin poisoning, and changes in the cholinesterase levels of blood and brain have been followed.

Materials and Methods

Mono*iso*nitrosoacetone, diacetylmonoxime, and pyridine-2-aldoxime methiodide were synthesized according to the methods of Green and Saville (1956). Oxime solutions and sarin dilutions were always prepared immediately before use, the former in distilled water and the latter in 0.89% NaCl. Atropine sulphate and urethane were dissolved in distilled water and stored in the refrigerator until required. In those tests involving treatment with oximes, atropine was used as an adjuvant to pyridine-2-aldoxime methiodide only.

Oximes and urethane were injected intraperitoneally; sarin and atropine subcutaneously. The dissolved substances were injected in the following volumes/200 g. of rat: diacetylmonoxime, 0.6 ml.; mono*iso*nitrosoacetone, 0.48 ml.; pyridine-2-aldoxime methiodide, 2.0 ml.; atropine, 0.2 ml.; urethane, 2.0 ml.; and sarin, 0.2 ml.

Male albino rats weighing 190 to 200 g. were used in all experiments. Blood specimens were withdrawn by cardiac puncture from rats which had died or been killed and from the tail vein in surviving animals when serial samples were required.

Cholinesterase assays were commenced as rapidly as possible after removal of tissues from the animal, and parallel control tests were performed in order to exclude the influence of *in vitro* artefacts of inhibition or reactivation. The unit of activity for determinations by the method of Michel (1949) is expressed as 100 times the decrement of *p*H observed during 60 or 90 min., and, by the manometric method, as μl. of CO_2 evolved/30 min.

Brains were removed from the rats immediately after death and were at once prepared for enzyme determination. They were weighed and then homogenized under uniform conditions. The interval between the time of death and the start of cholinesterase assay was 7.5 min.

Cholinesterase activity of whole blood, erythrocytes, plasma, and of most brains was estimated by the electrometric method of Michel (1949). Muscle and a few brain activities were measured by the Warburg manometric technique. The substrates used were methacholine chloride, butyrylcholine chloride, and acetylcholine chloride. Their final concentrations in the reaction mixture were 0.03 M, 0.03 M, and 0.015 M respectively. Methacholine was utilized to measure the activity of the brain and erythrocyte enzymes, butyrylcholine that of plasma, and

acetylcholine to estimate whole blood cholinesterase.

Oxime in blood was determined by the method described by Askew, Davies, Green, and Holmes (1956).

Results

The Clearance of Monoisonitrosoacetone and Diacetylmonoxime from the Blood.—After a single injection of mono*iso*nitrosoacetone or diacetylmonoxime, maximum concentrations of the oximes in rat blood were reached within 10 to 15 min. These then fell rapidly and at the end of 3 hr. were only 30% and 20% respectively of their highest levels (Fig. 1).

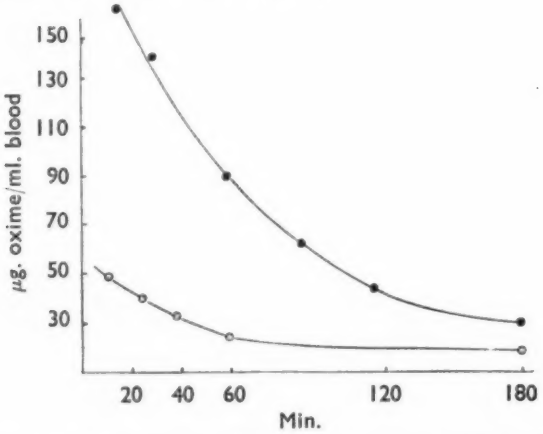

FIG. 1.—The concentration of mono*iso*nitrosoacetone and diacetylmonoxime in rat blood after the intraperitoneal injection of 35 mg./kg. and 150 mg./kg. respectively. Each value is mean of estimations on between 2 and 4 animals. ●: diacetylmonoxime. O: mono*iso*nitrosoacetone.

The "Protection" of Blood and Brain Cholinesterase by Monoisonitrosoacetone and Diacetylmonoxime.—Two groups of rats were given equimolar doses of mono*iso*nitrosoacetone or diacetylmonoxime 10 min. before the administration of 0.2 mg./kg. of sarin. For comparison, three sets of controls received one of the oximes or sarin alone.

All the animals injected with alkyl phosphate only died in less than 10 min., but the oxime controls and protected rats survived and were killed at times corresponding to the deaths of the sarin controls.

A small inhibition of brain cholinesterase was observed in the diacetylmonoxime controls, but otherwise the oximes, when used alone, had no effect upon either blood or brain enzyme (Table I).

After either of the oximes, the cholinesterase level of blood and brain was higher in pretreated rats than in the sarin controls. Mono*iso*nitrosoacetone appeared to protect blood cholinesterase to a greater extent than diacetylmonoxime although this protection was still only about 50%. Diacetylmonoxime, however, had the greater effect upon the brain enzyme; thus in those animals previously treated with this oxime, activity did not differ from the normal value and there was little variation between animals. After treatment with mono*iso*nitrosoacetone individual variation was marked and values varied from 25 to 90% of the normal values. The mean enzyme level was more than 50% of normal compared with 5% observed at death in the sarin controls. Two of the rats treated with mono*iso*nitrosoacetone exhibited signs of alkylphosphate poisoning, and it was subsequently found that their brain cholinesterase levels were the two lowest (33 and 54 units) of the group.

The Reactivation of Cholinesterase by Monoisonitrosoacetone and Diacetylmonoxime

Blood.—Reactivation of blood cholinesterase by mono*iso*nitrosoacetone was studied in the following manner: sixteen rats were each given sarin (0.2 mg./kg.) and 1 min. after signs of poisoning were evident (3 to 5 min. after injection) eight were killed whilst the remainder received oxime (35 mg./kg.). Four of these were killed 1 hr. later and the remaining four after a further hour had elapsed. The mean enzyme activities of the

TABLE I

CHOLINESTERASE ACTIVITY IN BLOOD AND BRAIN OF RATS TREATED WITH OXIMES AND THEN INJECTED WITH SARIN

Mono*iso*nitrosoacetone (MINA) (35 mg./kg.) or diacetylmonoxime (DAM) (41 mg./kg.) was injected 10 min. before sarin (0·2 mg./kg.). Means (in units of cholinesterase activity) ± s.d. The numbers of animals used are given within the brackets. Unit: decrement in *p*H produced in 90 min. by 0·5 ml. whole blood or brain homogenate. For whole blood the means of the activities of the 3 groups (untreated, mono*iso*nitrosoacetone (MINA) alone, diacetylmonoxime (DAM) alone) agreed extremely well while the results with corresponding groups for brain showed surprisingly poor agreement (Analysis of Variance: $P<0.005$), the low mean of "DAM alone" (133) being responsible.

Tissue	Oxime	Untreated	Sarin Alone	Oxime Alone	Sarin and Oxime	Sig. of Prophylactic Effect	Sig. of Diff. Between Oximes
Whole blood ..	MINA	138±26·7 (4)	26±2·22 (4)	135±16·19 (4)	59± 8·29 (4)	$P<0.001$	$P<0.03$
	DAM			138±10·34 (4)	38±11·59 (4)	$P\simeq0.09$	
Brain	MINA	156± 9·87 (8)	7±6·16 (4)	161± 2·50 (4)	87±37·98 (8)	$P<0.002$	$P<0.01$
	DAM			133±13·96 (4)	158±13·03 (6)	$P<0.001$	

plasma and red cells at these times were markedly higher ($P<0.001$) in the animals treated with mono*iso*nitrosoacetone than in the controls. The maximum degree of reactivation (Table II) seemed to be reached in 1 hr., but the 1 hr. and 2 hr. activities did not differ significantly ($P>0.3$).

TABLE II

CHOLINESTERASE ACTIVITY IN BLOOD OF RATS INJECTED WITH SARIN FOLLOWED BY MONO*ISO*NITROSOACETONE

The oxime (35 mg./kg.) was given 1 min. after appearance of signs of cholinesterase inhibition following injection of sarin (0·2 mg./kg.). Means ± s.d. (the number of animals in brackets). Unit of cholinesterase activity: decrement in *p*H produced in 60 min. by 0·5 ml. of whole blood, red cells or plasma.

Tissue	Sarin Alone	Sarin and Oxime	
		1 hr.	2 hr.
Whole blood (Normal: 94 units)	10·13 ± 4·82 (8)	28 ± 7·23 (4)	23 ± 2·22 (4)
Red cells (Normal: 89 units)	5·38 ± 2·97 (8)	23 ± 4·72 (4)	22 ± 6·40 (4)
Plasma (Normal: 93 units)	9·75 ± 2·44 (8)	29 ± 5·57 (4)	32 ± 7·46 (4)

(1) Comparisons between the figures for "Sarin Alone" and "Sarin and Oxime" in each case obviously reach the level of high significance.
(2) There is no significant difference between corresponding means of the columns "1 hour later" and "2 hours later."

Similar studies were made using diacetylmonoxime and a sublethal dose of sarin, but, in these, serial samples were withdrawn from the tail vein. Four rats were each injected with sarin and 30 min. later three received diacetylmonoxime (150 mg./kg.), whilst the fourth served as a control. A progressive increase in the whole blood activity of the animals treated with diacetylmonoxime occurred, while that of the control remained low throughout the experiment (Table III).

TABLE III

CHOLINESTERASE ACTIVITY IN BLOOD OF RATS INJECTED WITH SARIN FOLLOWED BY DIACETYLMONOXIME

The oxime (150 mg./kg.) injected 30 min. after sarin (0·06 mg./kg.) (3 rats, control sarin only). Cholinesterase activity as % of normal level for each animal.

Min. After Injection of Sarin	Control	Treated Rats			Mean Inc.
		1	2	3	
15	14%	8%	17%	21%	15·0% ($P=0·020$)
60	12%	25% (17%)	28% (11%)	38% (17%)	
120	15%	39% (14%)	48% (20%)	50% (12%)	15·3% ($P=0·024$)

Brain.—In a parallel series of experiments, brain cholinesterase activity was found to be depressed to 9% of the normal value 1 min. after a lethal dose of sarin (0.2 mg./kg.). Mono*iso*nitrosoacetone (35 mg./kg.) injected at this stage restored the activity to about 20 to 25% of normal within 10 to 15 min., but subsequent recovery was slower and, in 2 hr., a maximum of 30% of the original activity was regained (Fig. 2). An equimolar

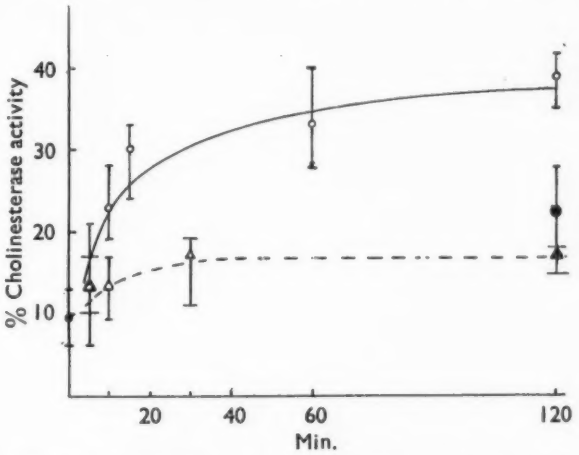

FIG. 2.—% reactivation of rat brain cholinesterase produced by oximes in sarin poisoning. Each value is mean (± range) of estimations upon 4 animals. Sarin controls at 0 min., 9%. O: mono*iso*nitrosoacetone (35 mg./kg.). ●: diacetylmonoxime (150 mg./kg.). Δ: diacetylmonoxime (41 mg./kg.).

dose of diacetylmonoxime (41 mg./kg.) was less effective, producing only 5% reactivation. The latter occurred in the first 10 to 30 min. and was not subsequently increased, although when the dose of oxime was raised to 150 mg./kg. reactivation was significantly higher at 2 hr. ($P<0.001$). A reasonable rectilinear relationship within the relevant range was obtained when cholinesterase units were plotted (Fig. 3) against log (log time in min.). The slope for treatment with diacetylmonoxime was not significant ($P>0.4$).

A second injection of mono*iso*nitrosoacetone 1 hr. after the first caused only an additional 6% increase in reactivation during the following hour, but the second dose caused marked side effects. This limited increase in reactivation was unexpected and raised the question as to whether it were possible to reactivate the remainder of the brain cholinesterase. Accordingly the brain was removed from a rat that had been poisoned with sarin and treated with mono*iso*nitrosoacetone. After homogenization, this was subjected to the further action of the oxime at 10^{-2}M and 38° which resulted in complete reactivation of the brain cholinesterase.

Spontaneous Regeneration of Cholinesterase.—The possibility that spontaneous regeneration of cholinesterase could account for the changes observed above was excluded by the following

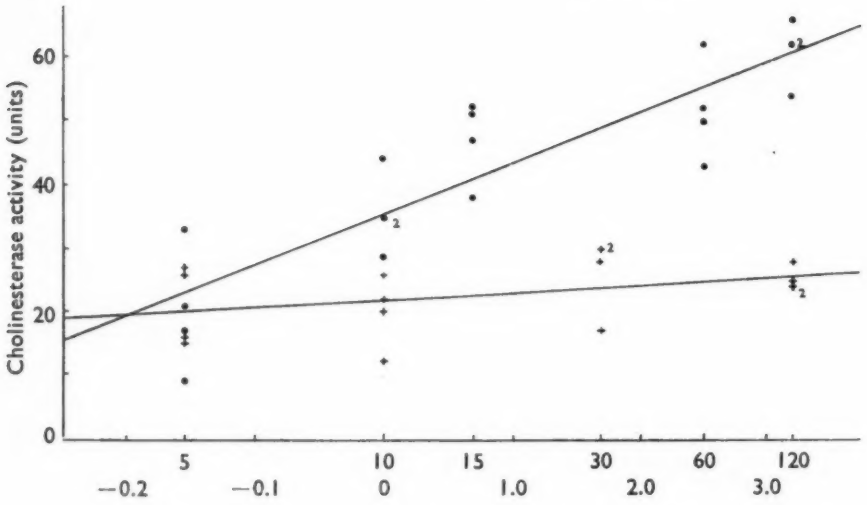

FIG. 3.—Rate of reactivation of rat brain cholinesterase by (a) mono*iso*nitrosoacetone(●: 35 mg./kg.) and (b) diacetylmonoxime (+ : 41 mg./kg.). Enzyme activity in units (*y*). Log (log time in min.) (*x*). Regression equations : (a) $y = 80{\cdot}23\ x + 35{\cdot}5$ (s.d. of slope: 20·8); (b) $y = 12{\cdot}15\ x + 22{\cdot}12$ (s.d. of slope: 16·0). Abscissa, time in min. above with log time below.

experiments. Five rats were anaesthetized with urethane (1.25 g./kg.) and artificially ventilated through a tracheotomy tube attached to a small Starling pump. They were then given atropine (17.5 mg./kg.) and 10 min. later sarin (0.2 mg./kg.). With this treatment they survived for 20, 45, 90, 90, and 120 min. The brain cholinesterase levels at death were 10, 11, 12, 21, and 6 units respectively, values which agreed well with the mean figure of 14 units found in sarin controls killed 1 min. after exhibiting signs of poisoning.

Reactivation of Cholinesterase by Pyridine-2-aldoxime methiodide.—Since pyridine-2-aldoxime methiodide is relatively ineffective in sarin poisoning, it was necessary to supplement its therapeutic action by giving atropine (17.5 mg./kg.) 5 min. before sarin (0.2 mg./kg.). With this exception the experimental plan was similar to that described when using mono*iso*nitrosoacetone.

The results (Table IV) showed that pyridine-2-aldoxime methiodide produced marked reactivation (30% and 50% respectively) of whole blood and skeletal muscle cholinesterase, but only a relatively small increase in brain (3%) and spinal cord (9%) activity.

TABLE IV

CHOLINESTERASE ACTIVITY IN BLOOD OF RATS INJECTED WITH SARIN FOLLOWED BY PYRIDINE-2-ALDOXIME METHIODIDE

Atropine (17·5 mg./kg.) was given 5 min. before sarin (0·2 mg./kg.); the oxime (100 mg./kg.) was injected 1 min. after appearance of signs of inhibition of cholinesterase. The rats were killed 1 hr. after injection of oxime. Means (in units) ± s.d. The numbers of animals used are given in brackets followed by mean expressed as approx. % of normal.

Tissue	Atropine+Sarin	Atropine+Sarin +Oxime (1 Hr. after Oxime)	Ratio (95% Fid. Limits)
Whole blood	2·75± 1·26 (4): 3%	31·50± 2·38 (4): 35%	11·5(7·8–21·0)
Skeletal muscle	17·33±11·00 (9): 15%	78·56±15·95 (9): 66%	4·5(3·1– 8·0)
Brain ..	10·25± 1·71 (4): 6%	13·75± 0·96 (4): 9%	1·3(1·1– 1·6)

*Control of Poisoning by Mono*iso*nitrosoacetone and Diacetylmonoxime.* — Sarin poisoning is characterized in the rat by muscular fasciculations, incoordination, violent convulsions, prostration, and gasping respiratory movements. Most of these signs were seen in the controls receiving 0.2 mg./kg. of sarin before death ensued, usually in less than 10 min. Following therapeutic treatment with mono*iso*nitrosoacetone, given 1 min. after the appearance of signs, however, the convulsions and muscular fasciculations ceased within 5 to 10 min., and 20 to 25 min. later there were no signs of poisoning. Diacetylmonoxime gave very much less relief at equimolar or higher doses, for although death was averted and major convulsions relieved within a few minutes, muscular fasciculations and minor convulsions remained until the animals were killed 2 hr. later.

DISCUSSION

The work described above was done to estimate the influence of oximes upon the level of cholinesterase in animals poisoned with sarin and to see if such changes could be related to the clinical picture. The therapeutic administration of mono*iso*nitrosoacetone reactivated the inhibited brain cholinesterase, and this occurred fast enough to explain the general improvement in the condition of the animal. The weaker therapeutic potency of diacetylmonoxime and the ineffectiveness of pyridine-2-aldoxime methiodide could also be related to their lesser power of brain reactivation.

If, however, the oximes were given before poisoning, no inhibition of brain cholinesterase occurred after diacetylmonoxime although blood activity was markedly depressed. This, also, was reflected in the clinical picture since the animals did not exhibit signs of sarin intoxication. Under the same conditions treatment with mono*iso*nitrosoacetone resulted in less inhibition of blood cholinesterase but greater inactivation of the brain enzymye as compared with diacetylmonoxime treatment, and severe signs of poisoning were sometimes observed.

The prophylactic and therapeutic conditions used here do not allow a simple interpretation of the way in which the oximes act under the defined circumstances. Whilst it is highly probable that reactivation was the operative process in those experiments in which the oxime was not given until signs of poisoning had developed, it is less likely that this is true when it was given before poisoning. Thus when diacetylmonoxime was used prophylactically neither signs of poisoning nor inhibition of brain cholinesterase were observed. Consequently, it seems that in this instance the brain enzyme was protected from inhibition, probably because the inhibitor was decomposed by direct action of the oxime. Mono*iso*nitrosoacetone, however, did not completely prevent inhibition and therefore the possibility that some concomitant reactivation occurred in this instance cannot be entirely disregarded.

The reason for the decreased rate of reactivation which commences 15 to 30 min. after a single dose of mono*iso*nitrosoacetone is not clear. However, three possible explanations may be considered. First, a second stage of inhibition (Hobbiger, 1955; Wilson, 1955; Jandorf, Michel, Schaffer, Egan, and Summerson, 1955; Davies and Green, 1955) occurs *in vivo* and no further reactivation is possible. Second, an inadequate concentration of oxime is maintained in the brain. Third, mono*iso*nitrosoacetone is unable to reach all the cholinesterase inhibited by the sarin. Since complete reactivation was achieved by subjecting inhibited brain homogenates derived from rats poisoned with sarin to the further action of mono*iso*nitrosoacetone, the first possibility can be eliminated, and it seems more likely that the limited reactivation is due to inadequate concentration of the oxime. If, however, the cholinesterase of the brain is distributed on either side of the neuronal membrane (Burgen and Chipman, 1952), the external cholinesterase being the portion responsible for the direct hydrolysis of acetylcholine (Koelle and Steiner, 1956), and it is assumed that sarin, but not mono*iso*nitrosoacetone, is able to penetrate to the internal portion *in vivo*, then the external cholinesterase alone will be reactivated. As the cholinesterase estimations in the present work are derived from total brain homogenates, this reactivation, even if maximal, would be apparent only as a part of the total normal activity.

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ANTIVIRAL ACTION OF DERIVATIVES OF ω-AMINOACETOPHENONE

BY

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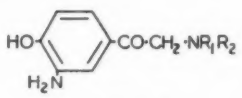
Numerous structural analogues of 3-amino-4-hydroxy-ω-methylaminoacetophenone were tested for their effect on the multiplication of influenza virus (FM_1 strain) in embryonated eggs, infected via the allantoic cavity. Antiviral activity was found in ω-aminoacetophenones containing an amino and hydroxyl group in the aromatic nucleus in the *ortho* or *para* positions to each other. The most powerful antiviral activity was found in the series of ω-alkylamino-5-amino-2 : 4-dihydroxyacetophenones. Derivatives of acetophenone with other substituents in the aromatic nucleus or in the aliphatic chain were without activity. *In vitro*, several analogues inactive in the egg test, as well as those which were active, exerted a virucidal and to a lesser extent a haemagglutinin-destroying action. Antiviral action in the allantoic test could not be prevented or inhibited by simultaneous administration of reducing substances, or of some amino-acids or vitamins. No inhibition of virus multiplication occurred in embryonated eggs infected via the yolk sac, or in mice infected intranasally with FM_1 virus. The activity in the allantoic test could be explained by the virucidal action of the compounds on the virus present in the allantoic fluid. No satisfactory interpretation of the empirical relationship between chemical structure and antiviral activity could be found.

In recent years numerous substances with antiviral properties have been reported. There are excellent reviews of this field by Matthews and Smith (1955) and Hurst and Hull (1956). However, none of the substances hitherto found to inhibit the multiplication of viruses in experimental host systems deserved clinical trial. The larger part of the investigations reported aimed at finding compounds interfering with the multiplication of the influenza viruses. These viruses were preferred for therapeutic trials because they are easy to handle and their multiplication can be determined fairly well. Moreover, the diseases caused by the influenza viruses have important medical and economic aspects.

In spite of much progress in the study of the multiplication of the influenza virus, the insight gained is not yet adequate for the establishment of a relationship between chemical structure and activity in antiviral substances. Therefore, it is still worth while to test compounds chosen arbitrarily for anti-influenzal activity. Screening tests of several hundreds of such compounds revealed that 3-amino-ω-*tert.*-butylamino-4-hydroxyacetophenone (A-5) inhibited the multiplication of influenza virus in embryonated hen eggs. A number of derivatives of phenol, benzoic acid and acetophenone having common structural features with A-5 proved to be inactive. This paper reports the results of an investigation intended to find other, possibly more active, derivatives of acetophenone. The empirical relationship between chemical structure and antiviral activity and the mode of action of these inhibitors of viral multiplication were also studied. The results obtained with A-5 suggested investigation of compounds with the general formula:



Group A

In this formula R_1 and R_2 represent a hydrogen atom or an alkyl, *cyclo*alkyl, or arylalkyl group. The other compounds of which the antiviral activity was investigated are structural analogues of these members of group A. Attention was paid to the influence of other substituents in the aromatic nucleus and to variation of the aliphatic chain.

Methods and Materials

Virus.—The FM_1 strain of influenza A′ virus used (World Health Organization Notation Influenza A-USA-47) had between 30 and 90 mouse passages and between 1 and 40 egg passages. We prepared our seed virus by the usual methods (Liu, Malsberger, Carter, Sanctis, Wiener, and Hampil, 1957).

Eggs.—Embryonated eggs of White Leghorns or Rhode Island Reds incubated for 11 days at 37° were used.

Virus Determination

Haemagglutination Titre.—The haemagglutination titre (HA) of an allantoic fluid was determined as described by van de Veen (1950). The titre is expressed as the logarithm of the final dilution with complete or partial haemagglutination. When calculating the logarithm, the interval between two successive two-fold dilutions was divided into four equal logarithmic intervals. Values lower than 1.2 were not determined exactly.

Infectivity.—Infectivity titrations were carried out as described by Rasmussen and Stokes (1951). The negative log ID50 was calculated by the method of Reed and Muench (1938). The standard deviations of the values so obtained were calculated according to Pizzi (1950).

Compounds

Details of the synthesis of the hydrochlorides and sulphates of the ω-aminoacetophenones are given elsewhere (Brug, 1958). The compounds were injected into the allantoic cavity in 0.2 ml. of a solution of 2, 1, 0.5, or 0.25×10^{-4} mole/ml. (4, 2, 1, and 0.5×10^{-5} mole/egg). Whenever possible they were dissolved in the required concentration in saline. If necessary HCl or NaOH was added. A *p*H near 7 was preferred but not obligatory. Solutions were sterilized by filtration. Poorly soluble compounds were suspended in 2% sterile carboxymethylcellulose gel. These manipulations were performed in sterile mortars under ultra-violet light in an aseptic cabinet.

Routine Test

For each concentration of the compound 6 eggs were injected with the solution or suspension mentioned above, in the allantoic sac. After 1 hr. the eggs were inoculated with 0.1 ml. of a 10^{-4} dilution of a virus suspension with log haemagglutinin titre $\geqslant$ 2.4. After incubation for 48 hr. at 36° the eggs were candled and dead ones discarded. 1 ml. portions of the allantoic fluid of each egg were harvested and pooled for each group. The haemagglutinin titres of these pools were determined. If doses of 0.5×10^{-5} mole/egg were too toxic, we tested lower concentrations. A compound was considered active and investigated further if in repeated experiments the difference between the log haemagglutinin titre of allantoic fluid from control eggs and the log titre of allantoic fluid from eggs treated with the compound under trial was 0.6 or more.

In Vitro *Tests*

Compounds giving a positive result in the routine test were investigated *in vitro*. The compound was dissolved in virus-containing allantoic fluid of high titre in a concentration equal to the lowest inhibitory concentration in the egg; the volume was taken as 10 ml., being the approximate amount of allantoic fluid in the egg. The log haemagglutinin titre and negative log ID50 of this mixture were determined immediately after mixing, and after 5, 24, 48, and occasionally 72 hr. incubation at 37°. As a control we used the same virus-containing allantoic fluid diluted with an equal volume of saline.

Results

*p*H *of Injected Solutions and Virus Multiplication*

Most compounds were brought into solution by adding an equivalent of HCl or NaOH. Some of the resulting solutions, as well as those of some acid or basic substances, had *p*H values below 5 or above 8. The buffering capacity of allantoic fluid in the region of *p*H 6 to 7 was determined to be about 0.008 van Slyke units. This value is in accord with the estimate obtained by Candles and Romanoff (1954). The low value of the buffering capacity made it necessary to check the influence of *p*H on the multiplication of influenza virus. To lower the *p*H of allantoic fluid a value near 6, about 1.5×10^{-5} equivalents of HCl must be added to 1 ml. of allantoic fluid. In the normal egg tests, we added no more than 0.4×10^{-5} equivalent of acid ml. of allantoic fluid. Fauconnier (1955) found that the multiplication of influenza A (PR 8 strain) in embryonated eggs was inhibited if the *p*H of allantoic fluid was 6 or less. Alkaline media also restrict growth of this virus (Ruffilli, 1955). Injection of 0.2 ml. saline made acid (*p*H 2 to 4) or alkaline (*p*H 10 to 12) by HCl or NaOH had no significant influence on the multiplication of influenza FM_1 as measured by the haemagglutinin titre of allantoic fluid. The mean difference between the log haemagglutinin titres of experimental and control fluids was about 0.15 units in 32 experiments over the ranges stated.

Antiviral Activity

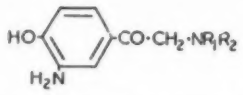
The effects of many derivatives of acetophenone substituted in the aromatic nucleus and in the aliphatic chain on the concentration of influenza FM_1 haemagglutinin in the allantoic fluid of infected eggs were determined. The compounds which caused a significant decreased concentration of haemagglutinin after an incubation period of 48 hr. are listed in Table I.

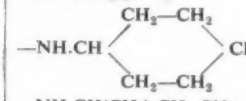
TABLE I

ANTIVIRAL ACTIVITY OF ω-AMINOACETOPHENONES

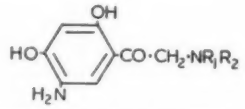
0·2 ml. of a solution of the compound was injected in allantoic cavity of 6 embryonated eggs. After 1 hr. eggs were infected with 0·1 ml. seed virus (influenza FM_1). After incubation for 48 hr. at 36° haemagglutination titre of pooled allantoic fluids was compared with that of pooled fluid from 6 control eggs infected with same amount of virus. Mean differences shown are based on at least 2 experiments.

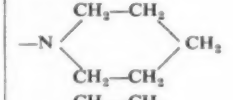
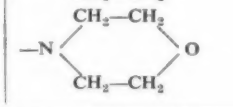
Group A



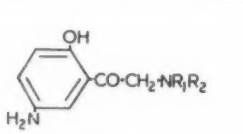
No.	$-NR_1R_2$	M. Conc. 4×10^{-5}	2×10^{-5}	1×10^{-5}	0.5×10^{-5}
A-1	$-NH_2$	1·1	0·9	0·6	0·3
A-2	$-NH.CH_3$	1·3	0·7	0·5	0·2
A-3	$-NH.C_2H_5$	1·6	0·7	0·2	0
A-4	$-NH.CH_2.CH(CH_3)_2$	>1·7	1·2	0·7	0·3
A-5	$-NH.C(CH_3)_3$	>1·7	1·6	0·7	0·4
A-6	$-NH.[CH_2]_3.CH_3$	1·9	1·1	0·7	0·2
A-7		>1·4	1·4	0·5	0·3
A-8	$-NH.CH(CH_3).CH_2.CH_2.C_6H_5$	1·6	1·6	0·9	0·4

Group F



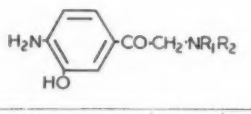
F-1	$-NH.CH_3$	>1·7	>1·7	1·5	0·3
F-2	$-N(CH_3)_2$	>2·0	>2·0	0·82	0·45
F-3	$-NH.CH(CH_3)_2$	>1·7	>1·7	1·4	0·15
F-4		1·5	1·5	0·6	0
F-5		1·6	1·6	0·6	−0·2

Group O



O-1	$-NH_2$	0·6	0·3	0·2	0
O-2	$-NH.C(CH_3)_3$	2·2	0·7	0·3	0·1

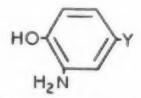
Group P



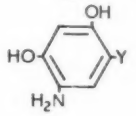
P-1	$-NH_2$	0·6	0·4	0·1	0·1
P-2	$-NH.C(CH_3)_3$	1·0	0·5	0·2	−0·1

Negative results were obtained when the substituted phenyl group of the compounds listed in Table I was replaced by the following groups: *p*-hydroxyphenyl (10), *m*-aminophenyl (4), 2:4-dihydroxyphenyl (7), 3:4-dihydroxyphenyl (7), 2-amino-4-hydroxyphenyl (4), 4-hydroxy-3-nitrophenyl (13). The numerals in parentheses denote the number of primary, secondary and tertiary ω-aminoacetophenones tested in each group. Methylation of the hydroxyl group or acetylation of the amino group in the aromatic nucleus of compounds A-2 and A-5 also caused a loss of antiviral activity. The active compounds of groups A, O, and P had a primary or secondary amino group in their aliphatic chain. Compounds of these types that had a tertiary amino group or an acetylated amino (acetamido) group proved to be inactive. On the other hand compounds of group F with a tertiary amino group in the aliphatic chain were highly active. The specificity of the structure of the aliphatic chain for the antiviral activity of ω-aminoacetophenones with appropriate substituents in the aromatic nucleus was illustrated by the negative results obtained with other compounds of the general types I and II below. In these formulae the symbols R_1 and R_2

I



II



I Y= $-H$, $-CH_3$ $-OH$, $-NH_2$, $-CHO$, $-CO_2H$, $-SO_3H$ $-CO.CH_2R_1$(6), $-CO.CH(CH_3).NR_1R_2$(8) $-CO.CH_2.CH_2.NR_1R_2$(6), $-CO.CH_2OR_1$(4)

II Y= $-H$, $-CO_2H$, $-CO.CH_2R_1$(2), $-CO.CH_2.CH_2.NR_1R_2$(2)

represent a hydrogen atom or an alkyl, *cyclo*alkyl, arylalkyl or saturated nitrogen-containing heterocyclic ring. The numerals in parthentheses give the number of compounds tested in each series. The compounds where Y represents a hydrogen atom or a methyl, hydroxyl or amino group were highly toxic for the chick embryo. Doses of about $1/8\times10^{-5}$ mole/egg were lethal. The remaining compounds, all having a strong electron attracting group at Y, had an LD50 $\geqslant 4\times10^{-5}$ mole/egg.

The apparent antiviral effects shown by the compounds listed in Table I are not due to interference of these compounds with determinations of the haemagglutinin titres of the viral suspensions harvested from the eggs. There was no significant difference between the titres of influenza FM_1 suspensions diluted with equal volumes of 0.02 M solutions of A-2 or A-5 and suspensions diluted

with an equal volume of saline. Breakdown products of A-2 or A-5 formed during the incubation period also did not disturb the determination of the titres. Table I suggests that the strength of the antiviral action in the A-series becomes greater with an increasing number of carbon atoms attached to the secondary nitrogen atom of the aliphatic chain. A comparison of the results obtained with compounds of groups A and F suggests that the latter compounds are more active than corresponding numbers of group A. This suggestion was confirmed by calculation of the relative potencies of two compounds by means of a (4×4) factorial scheme (Philippe, 1955). Taking the activity of A-2 as unity the 95% confidence limits of the calculated activity of A-5 are 2.0 to 3.9; of A-7, 2.3 to 4.6; and of F-3, 2.5 to 4.8.

A determination of the negative log ID50 of the allantoic fluids harvested from eggs incubated 48 hr. after infection with FM_1 virus and injection of the compounds confirmed the conclusions about activity based on measurement of the log haemagglutinin titre (Table II).

For closer investigation of antiviral activity most experiments were made with A-5 and F-3 as representatives of the inhibiting compounds. Table III shows that administering A-5 as early as 16 hr. before or as late as 24 hr. after inoculation of the seed virus still caused a significant, though smaller, decrease of virus growth.

TABLE II

EFFECT OF SUBSTANCES ON VIRUS GROWTH MEASURED BY THE NEGATIVE LOG ID50 AND LOG HAEMAGGLUTININ TITRE

Determinations made on a pool of allantoic fluid harvested after 48 hr. of incubation at 36° from eggs injected with 4×10^{-5} mole of a compound dissolved in 0·2 ml. saline, and 1 hr. later with 0·1 ml. of influenza virus FM_1, both being injected into allantoic cavity. Control eggs received 0·1 ml. of virus and 0·2 ml. saline.

Comp. See Table I	Log Titre Haemagglutinin	Difference from Control	Neg. Log ID50	Difference from Control
Control ..	3·0		8·5±0·3	
A-2	1·8	1·2	7·4±0·4	1·0±0·4
A-5	1·2	1·8	5·4±0·4	3·0±0·5
F-3	<1·2	>1·8	3·5±0·3	5·0±0·4

TABLE III

EFFECT OF INTERVAL BETWEEN INOCULATION OF VIRUS AND ADMINISTRATION OF A-5 ON CONCENTRATION OF HAEMAGGLUTININ IN ALLANTOIC FLUID OF EGGS MEASURED 48 HR. AFTER VIRUS INOCULATION (AT ZERO TIME) AND INJECTION OF 4×10^{-5} MOLE A-5

Interval (hr.)	−16	−2	−1	0	+1	+5	+24	Control
Log haemagglutinin titre ..	2·15	1·3	1·3	1·3	1·5	1·8	2·0	2·8

When the seed virus was less diluted and consequently the amount of virus injected into the allantoic fluid of the eggs was much larger than the amount inoculated in the standard procedure, the antiviral action of A-5 was much less pronounced. A dose of $4–10^{-5}$ mole A-5 showed no antiviral effect when an undiluted seed virus was used. Significant antiviral effects were observed with hundred-fold or higher dilutions of the seed virus.

A determination of the change in haemagglutinin concentration with time showed that increasing the dose of A-5 administered 1 hr. before inoculation of the virus delayed the moment at which virus growth became detectable in the allantoic fluid. Once detectable, the velocity at which the haemagglutinin subsequently increased appeared not to differ very much from that in control eggs (Fig. 1, curves *a*, *b*, *c*, and *d*). Determination of the negative log ID50 of the allantoic fluids at different times gave the same result (Fig. 2). A comparison of these curves with curves *f* and *g* in Fig. 1 suggests that the main effect of A-5 was an inactivation of a considerable part (99.9 to 99.99%) of the seed virus before attachment to susceptible cells. This suggestion is supported by the curve *e* in Fig. 1. Administration of A-5 24 hr. after the injection of the seed virus caused a subsequent rapid drop of the

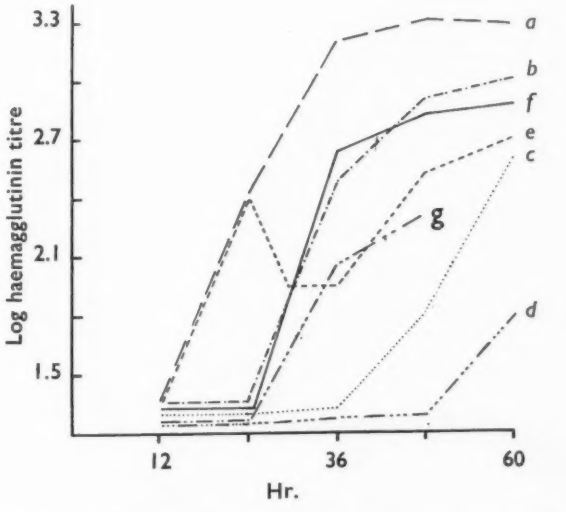


FIG. 1.—Changes with time of log haemagglutinin titre of pooled allantoic fluids from six eggs incubated at 36° after injection into allantoic cavity, in (*a*) to (*e*) inclusive, of 0·1 ml. of 10^{-4} standard virus suspension, and: *a*, no other injection; *b*, 10^{-5} mole A-5/egg 1 hr. before; *c*, 2×10^{-5} mole A-5/egg 1 hr. before; *d*, 4×10^{-5} mole A-5/egg 1 hr. before; *e*, $1·5 \times 10^{-5}$ mole A-5 24 hr. later; *f*, 0·1 ml. of 10^{-7} standard virus suspension only; *g*, 0·1 ml. of 10^{-8} standard virus suspension only.

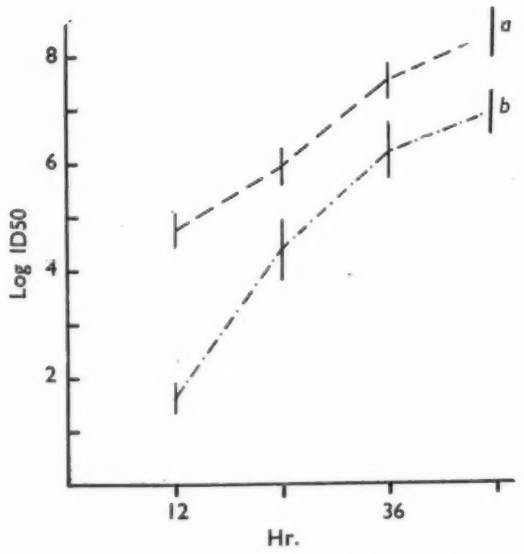


FIG. 2.—*a*, Increase of infecting power (negative log ID50) of pooled allantoic fluids from six eggs after injection of 0·1 ml. of 10^4 dilution of seed virus and 0·2 ml. of 0·9% saline. *b*, Increase of infecting power when 3×10^{-5} mole A-5/egg injected instead of saline. Incubated at 36°. Range marks indicate standard deviations.

haemagglutinin concentration. Further, this experiment indicated that the effective concentration of the inhibitor decreased rapidly.

An investigation of the direct action of the derivatives of acetophenone on influenza FM_1 was made *in vitro* (Tables IV and V). Compounds were added to virus-containing allantoic fluids in concentrations about equal to those used in the antiviral egg test. Incubation of these mixtures at 37° decreased their haemagglutinating and infecting power. The direct action on infecting power appeared to be more intense than the direct action on haemagglutinating power. At 4° or at 15°, the virucidal and the haemagglutinin destroying action was less powerful. The virucidal action of the derivatives of acetophenone appeared to be much weaker than that of formaldehyde, which has no antiviral effect in the egg test. Several derivatives of ω-aminoacetophenone having no antiviral effect in the egg test appear to have a virucidal and a haemagglutinin destroying action *in vitro*. Some examples are shown in Table IV. The structural relationships observed for the egg test therefore are not valid for the *in vitro* tests.

The importance of the *o*- and *p*-aminophenol structure for antiviral activity in the egg test led us to believe that this activity might be connected with the easy oxidizability of these compounds. Therefore we investigated whether the administration of reducing substances at the same time would suppress the antiviral action. Separate experiments had shown that ascorbic acid and sodium bisulphite protect a neutral solution of A-5 from oxidation by air. These reducing substances influence neither the results of the egg test nor the action *in vitro*. (±)-Cysteine, (±)-methionine, (−)-tryptophan, (−)-histidine, nicotinic acid, riboflavine, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid and 3-amino-4-hydroxy-

TABLE IV

IN VITRO ACTION OF ω-AMINOACETOPHENONES AND ANALOGUES ON THE INFECTIVITY OF INFLUENZA FM_1 VIRUS SUSPENDED IN ALLANTOIC FLUID

I. 4×10^{-3} M solutions of compounds in infective allantoic fluid incubated at 37°. The values given are the negative log of ID50. II. 4×10^{-3} M solutions in infective allantoic fluids incubated for 48 hr. at 37°. Infectivity is expressed as fraction of 6 eggs apparently infected by injection of 0·2 ml. of the incubation mixture. III. As for II, but incubated at 4°.

	Comp.	Incubation Time (hr.)				Result in Egg Test
		0	5	24	48	
I	A-2	8·5±0·4	7·1±0·4	0·6		+
	A-5	8·0±0·3	5·6±0·3	<0·5		+
	F-3	7·7±0·5	3·5±0·4	⩽0·5		+
	ω-*tert.*-Butylamino-*p*-hydroxyacetophenone	8·4±0·3	6·4±0·3	1·2±0·2		−
	4-Hydroxy-ω-methylamino-3-nitroacetophenone	8·7±0·3	6·6±0·3	⩽0·5		−
	3 : 4-Dihydroxy-ω-*iso*propylaminoacetophenone	8·6±0·3	5·5±0·3	⩽0·5		−
	Phenol	7·7	7·2	6·0	3·4	−
	Formaldehyde	5·5	−0·5	⩽−0·5		−
	Control	8·5±0·4	8·2±0·4	5·6±0·3		
II	A-5	—	5/6	0/6	0/6	+
	F-3	—	6/6	2/6	0/6	+
	3-Acetamido-4-hydroxy-ω-*iso*propylaminoacetophenone	—	5/6	1/6	0/6	−
	3-Amino-4-hydroxy-ω-piperidinopropiophenone	—	4/4	5/5	0/6	−
	Control	—	6/6	6/6	0/6	−
III	A-5	—	5/5	5/5	—	+
	F-3	—	6/6	4/4	—	+
	Control	—	6/6	6/6	—	−

TABLE V

IN VITRO ACTION OF ω-AMINOACETOPHENONES ON HAEMAGGLUTINATING POWER OF SUSPENSIONS OF FM_1 VIRUS IN ALLANTOIC FLUIDS

Compounds (4×10^{-3}M) dissolved in allantoic fluids containing virus.

Comp.	Incubation Temp.	Log Haemagglutinin Titre After				
		0 Hr.	24 Hr.	72 Hr.	120 Hr.	168 Hr.
Control	37	3·0	2·8	2·8	2·9	2·9
A-5	37	2·9	2·6	2·4	2·0	1·6
A-5	15	3·0	2·9	2·8	2·8	2·6
A-5	4	2·9	3·0	2·8	2·9	2·7
F-4	37	2·9	2·0	1·6	1·2	1·2
Phenol	37	2·4	2·7	2·8	2·8	2·7
Formaldehyde	37	2·4	2·7	2·7	2·6	2·7

benzoic acid were also tried as antagonists of the antiviral action of A-2 or A-5. They failed to show any effect.

Tests in Other Host Systems

Injection of 4×10^{-5} mole of F-3 or of 2×10^{-5} mole of F-4 into the yolk sac of the embryonated egg, followed 4 hr. later by infection of the yolk sac with 0.2 ml. of virus-containing allantoic fluid with a haemagglutinin titre < 2.6 diluted a thousand times failed to show any effect on the mean survival time of the embryo.

In a test with Swiss albino mice, daily subcutaneous injection of 50 mg./kg. of A-5 failed to prevent infection, and also failed to protect the animals against the consequences of intranasal infection with $3 \times$ LD50 influenza FM_1 virus.

Discussion

Inhibition of multiplication of influenza virus FM_1 in embryonated eggs by derivatives of ω-aminoacetophenone must be ascribed to their virucidal action. On the other hand substances with chemical structures closely related to those of the compounds active *in ovo* are equally virucidal *in vitro*, but have no effect on virus growth in the egg.

These different effects cannot be explained. During the initial period after administration of drug and virus to the egg, the concentration of virus in the allantoic fluid is very low and nothing is known about possible differences in virucidal action at these low concentrations. The experiments *in vitro* indicate that the virucidal action of the derivatives of acetophenone is not very effective. Therefore it seems reasonable to suggest that, in view of the long incubation period of 48 hr., differences in the rate at which these virucidal structures disappear from the allantoic fluid will play an important rôle.

In the molecules of the substances active in the egg test two important structural elements can be distinguished. These are the structures of *o*- or *p*-aminophenol and of ω-aminoacetophenone. Every change in each of these two structural elements reduced activity in the egg test. Other investigations have already shown that derivatives of phenol such as hydroquinone, *o*-aminophenol and *p*-aminophenol are virucidal for the influenza viruses (Kuroya, 1953; Hannoun, 1953; Groupé, Engle, Gaffney, and Hannaker, 1956; Spizizen, 1943; Kramer, Robbins and Smith, 1955). Virucidal activity has also been reported for acetophenone and some of its derivatives (Hartmann, 1954). These derivatives of phenol and of acetophenone, however, did not inhibit the development of influenza virus haemagglutinin in the allantoic fluid of infected embryonated eggs (Takemoto, Robbins, and Smith, 1954). Therefore it is interesting that the combination of these structural features in our compounds produced activity *in ovo*.

The inactivity of ω-aminoacetophenones with other substituents in the aromatic nucleus and the activity of those containing the *o*- and *p*-aminophenol structures irrespective of the position of the amino and hydroxyl group relative to the side chain indicate that specific properties of these structures are responsible for the antiviral action in the egg test. Both *o*- and *p*-aminophenols are easily oxidized. Preliminary experiments failed to show that the biological effects are connected with this property.

The introduction of a ketonic side-chain into the molecule of *o*- and *p*-aminophenol greatly reduces the toxicity of these substances for the chick embryo. The same effect is caused by other electron attracting groups. Changes of this kind also reduce the bactericidal and virucidal potency of derivatives of phenol (Takemoto *et al.*, 1954; Kramer *et al.*, 1955; Barber and Haslewood, 1945). The high specificity apparent from the investigation of several side-chain structures may be due in part to a differential reduction of the toxicities for host and virus.

These considerations, however, do not explain the special importance of the primary or secondary ω-amino group, present in representatives of groups A, O, and P. Chemically, primary and secondary α-aminoketones are unique in that they might form dihydropyrazines by an intramolecular condensation in the weakly basic allantoic fluid. It may be relevant that the carcinolytic (Lutz, 1955) and regeneration-inhibiting activity (Lehmann, Bretscher, Kühne, Sorkin, and Erne, 1950) of α-aminoketones has been ascribed to the formation of dihydropyrazines (Hinderling, Prijs, and Erlenmeyer, 1956). ω-Aminoacetophenones with a tertiary amino group in the aliphatic chain cannot be involved in this intramolecular condensation. The activity in the egg test of tertiary amino compounds of group F therefore makes the significance of this chemical possibility questionable unless one assumes that in the case of group F the substitution in the aromatic nucleus, which has the configuration of both *o*- and *p*-aminophenol is the determining factor. The greater antiviral action of compounds of group F than that of corresponding compounds of group A illustrates this possibility.

We wish to thank Professor Dr. Ir. J. Coops, of the Free University of Amsterdam, and Professor Dr. J. Mulder, of the State University of Leyden, for their helpful discussions. Part of this work was the subject of a thesis by one of us (J. B.) at the Free University of Amsterdam (1957). Mr. R. van Strik, of the Statistical Department of our Laboratories, kindly designed and analysed the experiment upon relative potencies of different preparations.

The technical assistance in the virological experiments of Mrs. R. Putman-Bloemendal and Miss A. Turien is thankfully acknowledged. Mr. J. van Dijk, Mr. H. Niewind, Mr. S. Polak, Mr. A. Vrijbergen, and Mr. E. H. Pol synthesized many compounds dealt with in this paper.

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THE UPTAKE OF ADRENALINE AND NORADRENALINE BY BLOOD PLATELETS OF THE PIG

BY

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Pig platelets contained 0.2 to 2.6 ng. adrenaline/10^8 platelets (4 experiments). Noradrenaline was not detected in them. When platelet-rich plasma was incubated at 37° with 1 or 10 μg. of added catechol amine/ml., the platelets continued to accumulate adrenaline for at least 120 min.; only about one-third as much noradrenaline as adrenaline was taken up. The concentrations of adrenaline taken up by different platelet samples at the end of incubation for 90 min. were proportional to the concentration of adenosine triphosphate in the platelets.

Mammalian blood platelets contain high concentrations of 5-hydroxytryptamine which they are able to take up from the plasma (Humphrey and Toh, 1954; Hardisty and Stacey, 1955). In some species, notably the rabbit, platelets also contain histamine (Humphrey and Jaques, 1954). The question arises whether the catechol amines which are found in blood (Holzbauer and Vogt, 1954) are likewise concentrated in the platelets. Results obtained with human blood by Weil-Malherbe and Bone (1954) and by Robinson and Stott (1958) suggested that this was so, although Valk and Price (1956) found no catechol amines in human platelets.

This paper reports experiments which show that the platelets of the pig contain adrenaline and that they are able to take up adrenaline from the plasma. We used pig platelets because we expected that the concentration of catechol amines in platelets would be low and because it was easy to obtain pig platelets in comparatively large amounts in blood brought from the slaughter-house: 1 l. of pig blood yielded up to 1 g. (moist weight) of platelets. A preliminary account of this work has appeared (Born and Hornykiewicz, 1957).

METHODS

All glassware with which platelets came into contact was coated with silicone (MS 1107). In the slaughter-house, the pig was stunned electrically and in 1 to 2 min. it was hung up by the hind-legs and bled by cutting the large veins in the neck. Samples of the freely falling blood were caught in a polythene container containing sufficient sodium citrate to give a final concentration of 0.36% (w/v).

The blood was taken to the laboratory and centrifuged at 500 *g.* and 1° for 20 min. to sediment the red and white blood cells. The supernatant plasma which contained the platelets ("platelet-rich plasma") was removed. Duplicate 0.1 ml. samples were mixed with 1.9 ml. of a solution containing 1% (v/v) formaldehyde in 3.6% (w/v) sodium citrate. The platelets in this diluted suspension were counted in haemocytometer chambers.

In some experiments, samples of platelet-rich plasma were then centrifuged at 6,000 *g.* for 20 min. at 1°. The sedimented platelets were extracted (see below) and the extracts were analysed for catechol amines and for adenosine triphosphate. The total time between the bleeding of the pig and the extraction of the platelets was 1 to 2 hr.

Incubation of Platelets with Added Catechol Amines.—Samples of platelet-rich plasma, about 30 to 40 ml., were incubated in centrifuge tubes in a water-bath at 37°. Catechol amines were added to some samples before incubating; the concentrations are given in the section on results. After incubation, the samples were centrifuged to sediment the platelets. In some experiments the platelets were washed with saline and centrifuged again. They were then extracted.

Adenosine triphosphate was extracted from the platelets with 10% (w/v) trichloracetic acid and determined by the firefly luminescence reaction (Strehler and Totter, 1954).

Catechol amines were extracted from platelets with acid ethanol by the method of Crawford and Outschoorn (1951). This method also extracted 5-hydroxytryptamine. The catechol amines were separated by paper chromatography (Vogt, 1952).

The system did not separate adrenaline from 5-hydroxytryptamine.

Noradrenaline was assayed by its effect on the blood pressure of the pithed rat. At first, adrenaline was assayed in the same way. However, Dr. Marthe Vogt pointed out to us that in such rats the blood pressure might be raised by the 5-hydroxytryptamine present in extracts of platelets. The quantity of 5-hydroxytryptamine in extracts of three samples of platelets, assayed on a strip of isolated rat stomach (Vane, 1957), was found to be 29, 37, and 42 ng./10^8 platelets. The presence of 5-hydroxytryptamine in such amounts would make the determination of adrenaline inaccurate. However, in the experiments in which the uptake of adrenaline was followed we were concerned only with measuring the increase in the quantity of adrenaline in the platelets. For this reason the presence of 5-hydroxytryptamine did not affect the results. In addition, these results were confirmed in other experiments in which adrenaline was assayed on the isolated uterus of the rat by the method of de Jalon, Bayo and de Jalon (1945). Lysergic acid diethylamide (10 ng./ml.) was present in the fluid bathing the uterus in order to abolish any stimulating effect of 5-hydroxytryptamine on the uterus.

Results

Presence of Adrenaline in Freshly Isolated Platelets

When pig platelets were isolated in the way described, they contained adrenaline: the value of 5.8 ± 0.88 ng./10^8 platelets (mean $\pm$ S.E.) was given in the preliminary communication (Born and Hornykiewicz, 1957), but that figure was probably too high because the assays were made with the rat blood pressure. Four later determinations with the rat uterus gave 0.2, 0.5, 1.5, and 2.6 ng./10^8 platelets.

In four samples of platelets which contained adrenaline, noradrenaline was not detectable, and the concentration present was therefore less than 0.3 ng./10^8 platelets. The area in which the concentration of noradrenaline would have been expected was cut from the chromatograms and eluted. When the eluate was assayed on the rat blood pressure there was no effect. This confirmed the conclusion that there was no noradrenaline in the platelet extracts.

Uptake of Catechol Amines by Platelets

When adrenaline was added to platelet-rich plasma it was taken up by the platelets against the concentration gradient. The amount of adrenaline taken up increased for at least 120 min. (Fig. 1).

Table I shows that when platelet-rich plasma was incubated with adrenaline for 90 min., the amount of adrenaline taken up by the platelets increased with increasing concentrations of adrenaline, but the final concentration gradient decreased steadily.

When noradrenaline was added to platelet-rich plasma at concentrations of 1 μg./ml. or 10 μg./ml. the platelets took up only about one-third as much as adrenaline.

Effect of Washing Platelets with Isotonic Saline on their Content of Adrenaline.—In two experiments, adrenaline was added to platelet-rich plasma and, after incubation for 90 min., the platelets were centrifuged down in the usual way. In some tubes the sedimented platelets were extracted at once for adrenaline. In others they were suspended with gentle stirring in 20 to 30 ml. 0.9% (w/v) NaCl, centrifuged again and then extracted.

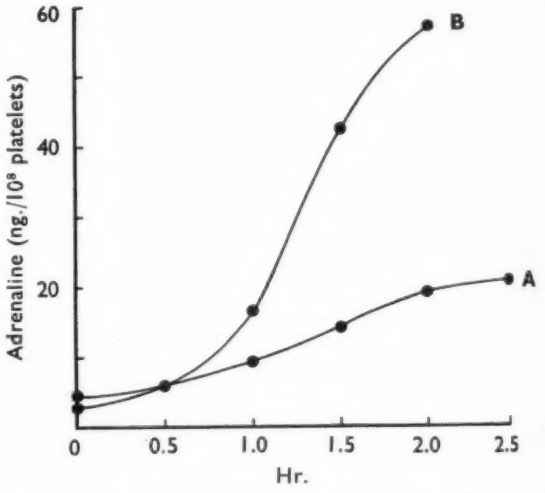

FIG. 1.—Uptake of adrenaline by pig platelets. Observations on platelet-rich plasma of two pigs. A: 1 μg./ml. of adrenaline added to plasma: adrenaline in platelets was assayed on rat blood pressure. B: 10 μg./ml. of adrenaline added to plasma: adrenaline in platelets was assayed on rat uterus.

TABLE I

UPTAKE OF INCREASING CONCENTRATIONS OF ADRENALINE ADDED TO PLATELET-RICH PLASMA OF THE PIG

Incubation: 90 min. at 37°. Average vol. of pig platelet: 12·3 μ^3.

Adrenaline Conc.			Platelet Conc./ Plasma Conc.
In Plasma	In Platelets		
(μg./ml.)	(ng./10^8 Platelets)	(μg./ml. Platelet Volume)	
1	14·2	11·5	11·5
10	60	49	4·9
50	216	176	3·5
250	678	552	2·2
500	918	746	1·5

Table II shows that washing with saline did not affect the amount of adrenaline in the original platelets nor in those incubated with 1 μg. adrenaline/ml. plasma. However, when the plasma had contained 10 μg. adrenaline/ml. the amount of adrenaline in the platelet sediment was reduced by about 12%.

TABLE II

EFFECT OF WASHING WITH ISOTONIC SALINE ON ADRENALINE CONTENT OF SEDIMENTED PLATELETS

Adrenaline Conc.		
Added to Platelet-rich Plasma (μg./ml.)	In Unwashed Platelets (ng./10^8 Platelets)	In Platelets Washed Once with Saline (ng./10^8 Platelets)
Nil	1·6	1·6
1	8·0	8·0
10	83·3	73·1

In two other experiments estimations were made of the amount of plasma trapped with platelets which had been centrifuged. This was done by determining the "inulin space" (Creese, 1954) in the pellet of platelets. From this the amount of adrenaline present in this volume of plasma was determined. It was calculated that, when the plasma contained 10 μg. adrenaline/ml., about 14% of the "platelet adrenaline" was accounted for by adrenaline outside the platelets. This suggested, therefore, that washing the platelets with saline merely removed plasma containing adrenaline and did not remove any adrenaline from the platelets themselves.

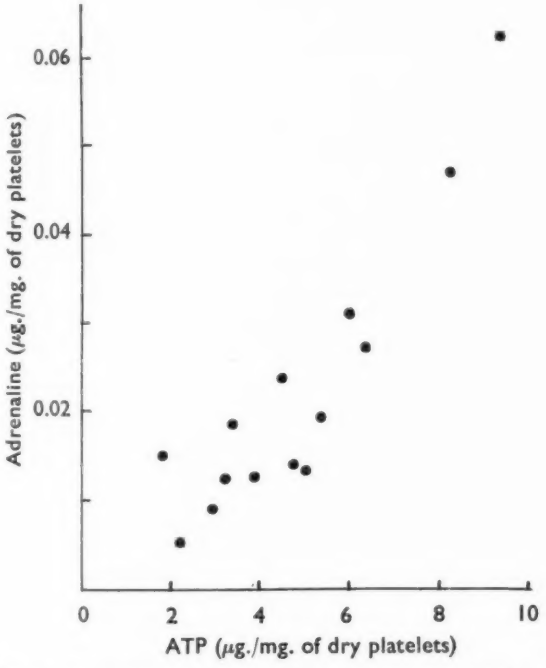

FIG. 2.—Proportionality between adenosine triphosphate (ATP) and adrenaline concentrations in platelets after addition to plasma of 1 μg. adrenaline/ml. and incubation at 37° for 90 min. $r=0.905$; $P<0.01$.

Proportionality Between the Amount of Adrenaline Taken Up by the Platelets and their Content of Adenosine Triphosphate.—Platelets were incubated for 90 min. in plasma to which 1 μg. adrenaline/ml. had been added, and adenosine triphosphate and adrenaline were determined in the platelets. It was found that there was a proportionality between the concentrations of adrenaline and of adenosine triphosphate in the platelets. This is shown in Fig. 2 in which the results are expressed on the basis of dry weight of platelets. The proportionality was also seen, but not so well, when the results were expressed in terms of the platelet count.

DISCUSSION

In these experiments pig platelets were found to contain adrenaline. On the basis of the amount of adrenaline in the platelets, pig plasma contained up to about 15 μg./l. This is much more than the concentrations of adrenaline found by Holzbauer and Vogt (1954) in the plasma of undisturbed, conscious dogs (between 0.04 and 0.25 μg./l.) and in one healthy man (below 0.06 μg./l.).

It is possible that the difference may be explained as follows. Whereas the samples analysed by Holzbauer and Vogt (1954) were obtained under resting conditions, platelets for use in the experiments described here were obtained from the blood of pigs which had been driven with some violence into the slaughtering pen, had then been stunned electrically and had finally been bled out from the veins in the neck. All these procedures are known to increase greatly the secretion of adrenaline from the suprarenal medulla into the blood.

It is not known whether platelets contain adrenaline when they are in the blood of the living pig. It took 1 to 2 hr. to isolate the platelets from the blood of the pig after it had been killed. The results on the rate at which adrenaline is taken up by platelets *in vitro* are consistent with the possibility that all the adrenaline which was found in the platelets was taken up by them during the time which elapsed between the death of the pig and the isolation of the platelets. The amount of free adrenaline, if any, left in the plasma was not determined.

It is interesting that the platelets contained adrenaline but no detectable noradrenaline. Moreover, when they were exposed to the same concentration of adrenaline and noradrenaline *in vitro*, they took up more adrenaline than noradrenaline.

The uptake of adrenaline by platelets was proportional to the amount of adenosine triphosphate in the platelets. In this the uptake of adrenaline by platelets resembles the uptake of 5-hydroxytryptamine (Born, Ingram and Stacey, 1958), an observation which led to the suggestion that in platelets amines are bound to adenosine triphosphate through their ionized groups. Work with granules which can be isolated from the cells of the adrenal medulla has made it likely that such compounds exist, because the granules contain both catechol amines and adenosine triphosphate in exceptionally high concentrations; moreover, the number of molecules of amine is probably just sufficient to satisfy all the acidic groups of the adenosine triphosphate (Hillarp, Högberg and Nilson, 1955; Blaschko, Born, D'Iorio and Eade, 1956). In the platelets this may be so in the case of 5-hydroxytryptamine (Born and Gillson, 1957) but not in the case of adrenaline. When platelets have taken up as much adrenaline *in vitro* as they can, they still contain far less adrenaline than adenosine triphosphate. If, therefore, an ionic bond is formed between the amine and adenosine triphosphate it is only one feature of a more complicated process. In pig platelets the preferential uptake of adrenaline over noradrenaline shows that the uptake is more specific than can be explained by ionic binding alone.

O. H. and A. S. would like to thank Dr. G. S. Dawes for the opportunity of working in the Nuffield Institute during the tenure by O. H. of a scholarship awarded by the British Council, to whom he is grateful, and while A. S. was Heydon Travelling Fellow of the University of Sydney.

We wish to thank Dr. W. D. McElroy for the fireflies, and Miss Janet Bricknell for technical assistance. We gratefully acknowledge the permission of the Editors of the *Journal of Physiology* to reproduce Fig. 2.

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A DEPRESSANT EFFECT OF ACETYLCHOLINE ON THE IDIO-VENTRICULAR PACEMAKER OF THE ISOLATED PERFUSED RABBIT HEART

BY

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In Langendorff preparations of the isolated rabbit heart, excision of the atria and severance of the atrio-ventricular bundle resulted in the onset of idio-ventricular rhythm at a rate much below the original sino-atrial rate. The mean decrease in rate in twelve experiments was 61%. Acetylcholine perfusion decreased the idio-ventricular rate still further. Physostigmine augmented this effect while atropine prevented or abolished it. Similar results were obtained with a rat heart. The results indicate that a cholinergic receptor mechanism is present at pacemaker sites in mammalian ventricles. However, when rabbit ventricles, beating under atrio-ventricular nodal rhythm or idio-ventricular rhythm, were cooled to the point of cardiac arrest, acetylcholine failed to cause reappearance of the cardiac beat.

Acetylcholine has been shown to affect the transmembrane atrial resting and action potentials in a number of species (Cranefield and Hoffman, 1958), and these effects present a basis for understanding the actions of this substance on rate, conduction velocity, and refractory period of atrial tissue. Effects on the transmembrane action potential of the frog ventricle have also been demonstrated, but experiments in which the effect of acetylcholine on the mammalian ventricular action potential was examined have proved negative (Brooks, Hoffman, Suckling, and Orias, 1955). Further, the generally accepted fact that the ventricle does not receive cholinergic innervation has been suggested as a reason for these negative results (Hoffman and Suckling, 1953; Brooks *et al.*, 1955). The experiments presented here indicate that acetylcholine can decrease the idio-ventricular rate of the isolated perfused rabbit and rat heart, and that this effect is augmented by physostigmine and is reversed or prevented by atropine. The effect of acetylcholine on isolated rabbit ventricles cooled to the point of cardiac arrest was also examined. The results of 19 experiments are described.

METHODS

Rabbit hearts were set up for Langendorff perfusion at 38°. The perfusion fluid used was that described by McEwen (1956). The heart rate was measured from the electrocardiogram, which was recorded by leads from the two ventricles. In a few early experiments, heart rate was counted with the aid of a stop-watch by observing ventricular contractions on a kymograph drum. For the production of atrio-ventricular nodal rhythm the atria and interatrial septum were excised at the level of the atrio-ventricular groove. In the experiments on idio-ventricular rhythm, the atrio-ventricular bundle was severed by an additional cut in the inter-ventricular septum. Drugs employed were added to the bottles containing the perfusion fluid. Drug concentrations are expressed as g./l. of the base. When hearts were cooled, this was accomplished by the gradual adding of tap water and ice to the water-bath in which the perfusion coil was immersed. The rate of cooling was about 1°/min.

RESULTS

Idio-ventricular Rhythm and the Effect of Acetylcholine

Table I shows the change in rate produced in the isolated perfused rabbit heart by excision of the atria and severance of the atrio-ventricular bundle. In 12 experiments, the mean heart rate dropped from 166 to 66 beats/min., a mean decrease in rate of 61%. In each of six of these experiments acetylcholine proved capable of decreasing the idio-ventricular rate still further and the magnitude of the effect increased with

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TABLE I

THE CHANGE IN HEART RATE PRODUCED IN THE ISOLATED, PERFUSED RABBIT HEART BY EXCISING THE ATRIA AND SEVERING THE ATRIO-VENTRICULAR BUNDLE

Sino-atrial Rate (Beats/Min.)	Idio-ventricular Rate (Beats/Min.)	Difference (Beats/Min.)	% Decrease
120	47	73	61
138	90	48	35
145	38	107	74
152	40	112	74
160	76	84	53
165	41	124	75
170	76	94	55
170	102	68	40
172	29	143	83
181	95	86	48
190	51	139	73
234	102	132	57
Mean 166	66	101	61
Range 120–234	29–102	48–143	35–83

TABLE II

THE EFFECT OF INCREASING THE ACETYLCHOLINE CONCENTRATION (WITH AND WITHOUT PHYSOSTIGMINE) ON THE IDIO-VENTRICULAR RATE OF THE ISOLATED, PERFUSED RABBIT VENTRICLE

Heart rates are given in beats/min. ACh = acetylcholine. The results on each horizontal line all refer to one experiment. The letters in brackets relate results from the same experiment. (1) Indicates that the response was prevented by atropine 10^{-7} during subsequent ACh perfusion; (2) that the response was reduced by atropine 10^{-7} during continued ACh perfusion; (3) that the effect was prevented by atropine 10^{-6} during subsequent ACh perfusion; (4) that the results were obtained in the presence of atropine 10^{-7}.

ACh Conc.	Without Physostigmine		Physostigmine 10^{-7}		Physostigmine 10^{-6}	
	Control Rate	% Decrease after ACh	Control Rate	% Decrease after ACh	Control Rate	% Decrease after ACh
10^{-7}	40 (a)	8				
	76	13	63	37	53	91 (1)
	95 (b)	34 (2)				
			82 (c)	100 (1)		
10^{-6}	36 (a)	17				
	63	17			53	100 (2)
	90	32			82	100 (2)
					60	100 (1)
10^{-5}	32 (a)	44				
	76 (b)	63 (3)				
	80 (b)	54				
	85 (c)	49 (4)				
	90 (c)	52 (4)				
					38	100 (3)

increasing concentrations. Physostigmine, which by itself reduced the rate slightly, markedly augmented the effect of acetylcholine. Cardiac arrest, not seen with a 10^{-5} concentration of acetylcholine, was observed with a 10^{-7} concentration of acetylcholine in the presence of physostigmine 10^{-7}. In concentrations of 10^{-7} and 10^{-6}, atropine prevented or abolished the acetylcholine effect even in the presence of physostigmine. These results are summarized in Table II. Similar

TABLE III

THE EFFECTS ON RHYTHMICITY OF PROGRESSIVE COOLING OF THE ISOLATED, PERFUSED RABBIT VENTRICLE

(2) This result was obtained from the heart with suffix (1) following a second period of cooling after rewarming to 38°.

	Temperature at which Contractions Ceased	Q_{10} for Heart Rate (38–28°)
Atrio-ventricular nodal rhythm	6·2	2·1
	6·8	
	12·5	2·0
	14·0	
	14·8	
Idio-ventricular rhythm	13·5	2·3 (1)
		2·9 (2)
	17·2	3·3
	18·0	2·7
	Mean 12·9	2·6
	Range 6·2–18·0	2·0–3·3

results with acetylcholine, physostigmine and atropine were observed in one experiment with the isolated perfused rat heart after excision of the atria and severance of the atrio-ventricular bundle.

The Effects of Cooling

In eight hearts, with atria excised (atrio-ventricular nodal rhythm), three of which had the atrio-ventricular bundle severed (idio-ventricular rhythm), progressive cooling resulted in cardiac arrest at a mean temperature of 12.9° (range 6.2 to 18.0). The Q_{10} for heart rate (the increase in heart rate for a rise of temperature of 10°) measured in five of these hearts over the temperature range 38° to 28°, had a mean value of 2.6 (range 2.0 to 3.3).

Table III summarizes the results. Acetylcholine in concentrations of 10^{-7}, 10^{-6}, and 10^{-5} failed to cause the reappearance of the cardiac beat during cardiac arrest at the low temperatures. Acetylcholine (10^{-7}, 10^{-5}), administered at low temperatures, but not low enough for cardiac arrest, produced cardiac slowing or cardiac arrest in each of three experiments.

DISCUSSION

The excision of the atria and section of the atrio-ventricular bundle to establish ventricular pacemaker activity in the isolated rabbit heart is an unphysiological procedure. However, it provided a series of hearts in which ventricular pacemaker activity, at rates well below the original sino-atrial rates, could be studied. The exact location of the pacemaker was unknown, but on anatomical grounds it could not have been atrial or nodal.

The findings that acetylcholine decreased the rate of ventricular pacemaker discharge, that physostigmine augmented this effect and that atropine prevented or abolished it clearly indicate the presence of a receptor mechanism for acetylcholine in the rabbit and rat ventricle. A report by Baker (1953) on the slowing of the idio-ventricular rate produced by acetylcholine in the isolated perfused human foetal heart suggests that a similar mechanism exists in the human ventricle.

Additional support for the idea that acetylcholine has a depressant effect on the rate of discharge of ventricular pacemakers comes from experiments on the isolated perfused rat heart. In this preparation, as in the rabbit heart, atrial excision and severance of the atrio-ventricular bundle caused a marked drop in rate consistent with the appearance of an idio-ventricular pacemaker.

However, in experiments on isolated perfused rat hearts beating under sino-atrial rhythm, acetylcholine and physostigmine perfusion for 8 min. caused immediate cessation of the beat without the establishment of an idio-ventricular pacemaker (Benforado, 1959). Such an effect points to inhibition of all cardiac pacemakers by acetylcholine.

Although it is generally accepted that the ventricles do not receive cholinergic innervation, this fact cannot be a basis for suggesting (Hoffman and Suckling, 1953; Brooks *et al.*, 1955) that the ventricular cell membrane is incapable of responding to acetylcholine. Numerous examples of responsiveness of non-innervated tissues to transmitter agents are available. For acetylcholine one of the best studied examples is the nerve-free gillplate of *Mytilus edulis* (Bülbring, Burn and Shelley, 1953). The responsiveness of nerve-free placental vessels to adrenaline was demonstrated by von Euler (1938).

That the isolated rabbit ventricle can liberate acetylcholine during perfusion is evident from the experiments reported by Day (1956). When recirculated perfusion fluid from rabbit hearts, with and without the atria excised, was assayed on the frog heart and on the frog rectus, two-thirds of the acetylcholine liberated was found to derive from the ventricles. This endogenous acetylcholine, which may play a rôle in impulse production in the ventricle, could account for the decrease in idio-ventricular rate with physostigmine described in the rabbit ventricle. Such effects on rate have also been noted in rabbit atria (Burn and Kottegoda, 1953).

In the experiments on cooling of rabbit ventricles during atrio-ventricular nodal and idio-ventricular rhythm, the Q_{10} values for changes in rate obtained over the range 38–28° were of the same order as those reported for isolated rabbit auricles (30 to 20°) by Marshall and Vaughan Williams (1956). In their experiments on auricles, acetylcholine was able to restart contractions after arrest at low temperatures. No such action was seen in the ventricles. The results in the atria were explained by the action of acetylcholine in increasing the resting membrane potential of cooled auricles, allowing non-conducted pacemaker potentials to be propagated. Confirmatory experiments, showing that acetylcholine increased the low diastolic membrane potential of cooled auricles, were reported by Marshall (1957). The negative results in the present experiments on the ventricles may have been due to the fact that pacemaker potentials were absent during cardiac arrest at low temperatures. However, since acetylcholine has no effect on the membrane potential of ventricular tissue there may be an alternative explanation. This is that it is unable to restore conduction in the cooled ventricle as it does in the cooled auricle. It would be misleading to infer from this that acetylcholine is without any effect on ventricle tissue. The present experiments indicate a depressant effect on pacemaker sites in the ventricle. Taken together with reports on ventricular arrhythmias induced by acetylcholine (Scherf and Schott, 1953) and the well-known effects of acetylcholine and vagal stimulation on the rate of discharge of atrial and atrio-ventricular nodal pacemakers, there appears to be a general responsiveness of cardiac pacemakers to this substance. However, the failure of Weidmann (1956) to observe any effect of acetylcholine on the rate of diastolic depolarization in Purkinje fibre pacemakers (species not specified) makes such a generalization untenable. The presence of an effect of acetylcholine on the action potential of frog ventricle and its absence in mammalian ventricle indicates a species difference. Such a difference may also exist between mammalian hearts. Differences in the effect of acetylcholine on the atrial resting membrane potential of various mammalian species (Cranefield and Hoffman, 1958) support this idea.

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THE RELATIVE POTENCIES OF SOME SUBSTITUTED SALICYLIC ACIDS AS METABOLIC STIMULANTS IN THE INTACT RAT

BY

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The effects of eighteen substituted benzoic acids on the rate of oxygen consumption have been studied in rats. 2:3-Dihydroxybenzoic acid, phthalic acid and 6-methylsalicylic acid were, at the doses used, inactive; *m*- and *p*-hydroxybenzoic acid, 2:4-, 2:5-, 2:6-, 3:4-, and 3:5-dihydroxybenzoic acid, *o*-aminobenzoic acid, salicyluric acid, salicylamide and 5-aminosalicylic acid decreased the rate of oxygen consumption. Only salicylic acid and *o*-, *m*- and *p*-cresotic acid (3-, 4- and 5-methylsalicylic acid respectively) increased the rate of oxygen consumption. Molar potency ratios of the cresotic acids as metabolic stimulants relative to salicylic acid were determined; *o*-cresotic acid was the most powerful with a ratio of 2.61, *m*- and *p*-cresotic acid had values of 1.78 and 1.89 respectively. Two possible explanations of the higher potencies of the cresotic acids were considered. No difference in the primary action of the drug was established by determining the effect on rate of oxygen consumption of a mixture of *o*-cresotic and salicylic acids. An alternative possibility was that the rates of detoxication and excretion of the cresotic acids differed among themselves and from salicylic acid. No such differences were found.

It is well established that sodium salicylate, in moderate dosage, increases the rate of oxygen consumption of experimental animals (Singer, 1901 ; Reid, 1957) and man (Barbour and Devenis, 1919 ; Cochran, 1952).

Meade (1954) found that only salicylic acid, among all the mono- and di-hydroxybenzoic acids, increased the rate of oxygen consumption of rats ; *m*-hydroxybenzoic acid was a depressant and the other acids were inactive. Hall, Tomich, and Woollett (1954) investigated a number of antirheumatic compounds and other substances chemically related to salicylic acid. They found that only salicylic acid and acetylsalicylic acid increased the rate of oxygen consumption of rats or mice ; 2:5- and 2:6-dihydroxybenzoic acid, *m*- and *p*-hydroxybenzoic acid and salicylamide were inactive.

The present work compares eighteen substituted benzoic acids with respect to their ability to stimulate oxygen consumption ; the relative potencies of the active compounds are compared and commented upon.

The rat was chosen as the most practical experimental animal, the smallest in which changes in oxygen consumption can be easily detected over 1 hr. periods.

METHODS

Oxygen consumption was measured with a closed-circuit manometric method described by Cameron (1958).

In the first experiments Wistar albino rats weighing 230 to 290 g. were used. The animals were paired for sex and weight, and in each run one animal received the test solution while the other received an identical volume of normal saline. The drugs were administered by intraperitoneal injection as solutions of the sodium salts at *p*H 7 to 9 ; the doses administered were the highest that could be tolerated.

Dose/Response Experiments. — The compounds used in these experiments were salicylic acid and *o*-, *m*-, and *p*-cresotic acid (3-, 4-, and 5-methylsalicylic acid respectively). Wistar albino rats of 170 to 250 g. weight were used and the animals were paired for sex and weight. As before, one animal was given the drug while the other received the same volume of normal saline. The difference in rate of oxygen consumption between the members of each pair ($\triangle O_2$, expressed in ml./hr. at s.t.p.) was determined. The animals were injected intraperitoneally with the sodium salts of the four acids in four doses ranging from an arbitrary low dose to the maximum dose generally tolerated by the rat. The order of observations was randomized, in respect of drug and dose, within a Graeco-Latin square and 12 estimates of $\triangle O_2$ (6 males, 6 females) were obtained for each drug at each dose.

The combined action of salicylic and *o*-cresotic acids was studied on Wistar albino rats of 200 to 250 g. weight. Six rats (three pairs of the same sex and weight) were used for each trial; one from each pair received normal saline while the other received an intraperitoneal injection of the sodium salt of salicylic acid, or the equipotent dose of *o*-cresotic acid, or a mixture of half of both, and $\triangle O_2$ was determined as before.

Time/Concentration Experiments. — Wistar albino rats of 150 to 350 g. weight were injected with the highest dose of salicylic acid or the cresotic acids used previously, and killed by decapitation at varying times after injection over a 16 hr. period. Eight determinations (4 males, 4 females) of the plasma drug concentration were made for each drug at each time. Plasma concentrations of salicylic acid and the three cresotates were determined by the method of Trinder (1954), which was found to be applicable to the methyl-substituted salicylic acids as well as to salicylic acid itself.

Results

The first experiments were made to determine which of eighteen compounds, related in their structure to salicylic acid, increased the rate of oxygen consumption of rats.

The composite hypothesis that the mean difference in the rates of oxygen consumption by the members of each pair of rats was zero (H_0) was tested against the single alternative that it differed from zero (H_1), using the sequential test proposed by Wald (1947) and the tables of Arnold (1951). Formally, in each case, where μ was the mean $\triangle O_2$ and σ^2 was its variance, it was decided whether $|\mu| < \delta\sigma$ or whether $|\mu| \geqslant \delta\sigma$. In these experiments δ, which determines the critical $\triangle O_2$, was assigned the value 1. The maximum probability of a decision in favour of either hypothesis when in fact the other was true was chosen as 0.05. Where H_1 was accepted, the sign of the mean $\triangle O_2$ was formally established from its 95% fiducial limits, since in no instance did these limits include zero. Table I summarizes these results.

It was previously decided that the results would be discarded if the animals were restless, convulsed, or died during the 60 min. experimental period. In fact, only one of over 400 estimations had to be discarded and this was due to death.

The rate of oxygen consumption of 164 control rats (weight range 230 to 290 g.) showed a nearly normal distribution. The mean rate of oxygen consumption was 420.9 ml./hr. ($\pm$60.3 : s.d.). A quality control chart confirmed that the experiments were in statistical control.

Only four compounds were found to increase the rate of oxygen consumption of rats. These compounds were salicylic acid and the three cresotic acids. Qualitatively these acids were very similar, the maximum doses tolerated were of the same order and the toxic effects observed with very high doses, such as hyperventilation and convulsions, were the same. The relative potencies of the cresotic acids as metabolic stimulants were therefore next compared with salicylic acid.

The dose/response curves were simultaneously determined and are shown in Fig. 1. There was for each compound an approximately linear relation between dose and response and there was no gross difference in intercept on the *y* axis. It seemed likely that these results would therefore permit a comparative slope ratio assay. Before proceeding to the analysis, homogeneity of variance was confirmed by Bartlett's test, $0.50 > P > 0.10$. The requirements for a valid slope/ratio assay are linearity and common intercept; the analysis of variance (Table II) shows that there was no significant deviation from linearity nor was there a significant component of variance due to difference in the intercepts on the *y* axis of the four regression lines.

TABLE I

EFFECT OF SALICYLIC ACID AND RELATED COMPOUNDS ON THE RATES OF OXYGEN CONSUMPTION OF WISTAR ALBINO RATS

$\triangle O_2$ is the difference in rates of oxygen consumption between paired treated and control rats (ml./hr.); H_0 is the hypothesis that the mean $\triangle O_2$ is zero, H_1 the alternative; n is the number of trials required for termination of the sequential test of H_0 against H_1.

Compound	Dose (mg.)	Mean O_2 Uptake of Control Rats (ml./hr.)	$\triangle O_2$: Mean and 95% Fiducial Limits (n)	Hypothesis Accepted
Salicylic acid ..	120	418·9	+53·5± 34·6 (10)	H_1
m-Hydroxybenzoic acid	500	457·7	−173·0±103·9 (6)	H_1
p-Hydroxybenzoic acid	500	409·8	−78·1± 42·1 (8)	H_1
2 : 3-Dihydroxybenzoic acid ..	100	430·0	−25·5± 55·9 (12)	H_0
2 : 4-Dihydroxybenzoic acid ..	300	456·6	−84·4± 44·5 (7)	H_1
2 : 5-Dihydroxybenzoic acid ..	500	474·3	−332·9± 71·6 (6)	H_1
2 : 6-Dihydroxybenzoic acid ..	200	438·4	−121·5± 71·0 (7)	H_1
3 : 4-Dihydroxybenzoic acid ..	500	454·8	−102·9± 67·4 (10)	H_1
3: 5-Dihydroxybenzoic acid ..	500	466·6	−104·5± 69·9 (7)	H_1
5-Aminosalicylic acid	100	399·5	−95·1± 55·4 (7)	H_1
Salicyluric acid ..	100	388·6	−46·9± 29·9 (8)	H_1
Salicylamide ..	50	409·4	−128·6± 46·8 (6)	H_1
o-Cresotic acid (3-methylsalicylic acid)	100	395·9	+176·1± 76·7 (7)	H_1
m-Cresotic acid (4-methylsalicylic acid)	100	385·1	+109·9± 33·6 (6)	H_1
p-Cresotic acid (5-methylsalicylic acid)	105	383·2	+186·6± 62·9 (8)	H_1
6-Methylsalicylic acid	24	442·6	+21·6± 61·7 (10)	H_0
o-Aminobenzoic acid	100	379·0	−68·7± 40·7 (7)	H_1
Phthalic acid ..	100	452·9	−13·6± 48·9 (9)	H_0

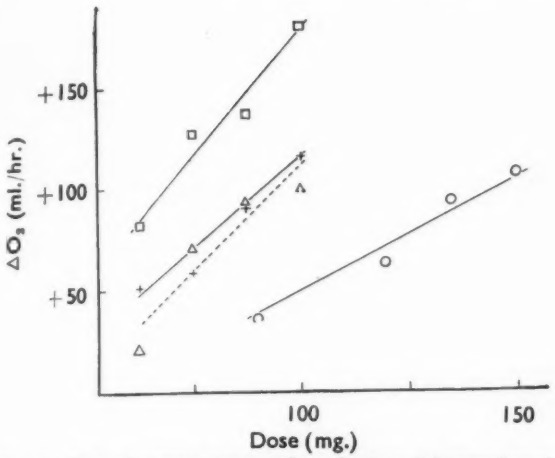

FIG. 1.—Rate of oxygen consumption of rats and intraperitoneal dose of salicylic acid (O); *o*-cresotic acid (□), *m*-cresotic acid (△ - - - - - -) and *p*-cresotic acid (+). For definition of ΔO_2 see text. Each point is mean of twelve determinations.

TABLE II

SLOPE/RATIO ASSAY OF SALICYLIC ACID AND THE CRESOTIC ACIDS: GENERAL ANALYSIS OF VARIANCE

Source of Variance	Sum of Squares	d.f.	Variance
Rectilinear regressions	148,493·81	4	37,123·45
Deviation from regressions ..	17,861·85	8	2,232·73
Between intercepts	3,672·35	3	1,224·12
Residual	704,831·22	176	4,004·72

The potency ratios of the cresotic acids with respect to salicylic acid were therefore calculated in molar units and are shown in Table III. The 95% confidence limits of these ratios were obtained by a maximum likelihood method (Silvey, personal communication).

TABLE III

MOLAR POTENCY RATIOS OF THE THREE CRESOTIC ACIDS RELATIVE TO SALICYLIC ACID AS METABOLIC STIMULANTS

Compound	Potency Ratio	95% Confidence Limits
o-Cresotic acid	2·61	2·50–2·72
m-Cresotic acid	1·78	1·69–1·87
p-Cresotic acid	1·89	1·79–1·99

In these experiments the rats weighed less than in the previous experiments. The rates of oxygen consumption of 252 control rats (weight range 170 to 250 g.) showed an apparently normal distribution, and a quality control chart confirmed that the experiment was in statistical control. The mean rate of oxygen consumption was 389.2 ml./hr. ($\pm$48.5: s.d.).

The distributions of the rates of oxygen consumption for the two series of experiments were similar, and although the rate of oxygen consumption depends on body weight there was no significant correlation between ΔO_2 and body weight for any drug or dose ($P>0.05$).

The preceding observations permit a quantitative study of the combined action of salicylic acid and the cresotic acids. Such experiments might point to a difference in the primary action of the drugs which in turn might explain the greater potencies of the cresotic acids.

TABLE IV

COMBINED ACTION OF SALICYLIC ACID AND *o*-CRESOTIC ACID

Results of the sequential tests of the hypothesis that the mean difference in ΔO_2 of rats treated with 150 mg. salicylic acid (S), 70 mg. *o*-cresotic acid (O), and a mixture of 75 mg. salicylic acid and 35 mg. *o*-cresotic acid (SO) were equal. Formally where each mean difference was μ and its variance σ^2, the hypothesis $H_0: |\mu| < 0{\cdot}7\sigma$ was tested against $H_1: |\mu| \geq 0{\cdot}7\sigma$, the probability of accepting the wrong hypothesis was 0·05.

Difference Tested	No. of Trials Required	Hypothesis Accepted	Mean Difference and 95% Fiducial Limits of the Mean
(S)–(O)	14	H_0	+1·6±38·5
(S)–(SO)	14	H_0	+6·0±41·6
(O)–(SO)	14	H_0	+4·4±43·5

The nature of the combined action of salicylic acid and *o*-cresotic acid was determined as described by Gaddum (1953). The effects of a selected dose of salicylic acid on the rate of oxygen consumption, an equipotent dose of *o*-cresotic acid, and a mixture of half the dose of each drug were compared. Table IV summarizes these results. In every case H_0 was accepted. Thus administration of a mixture of salicylic acid and *o*-cresotic acid gave a response which was indistinguishable from an additive effect, and therefore no difference in the primary actions of the two drugs was established by this experiment.

The possibility that differences in rates of degradation and excretion between salicylate and the

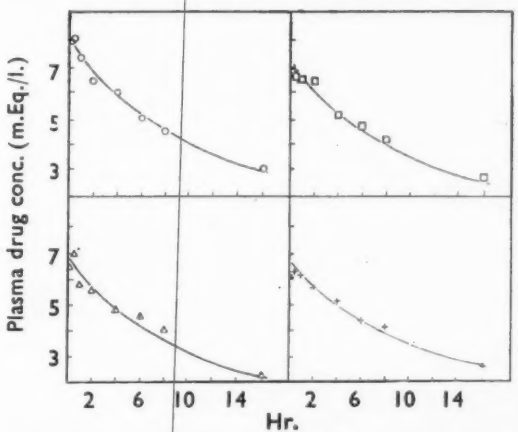

FIG. 2.—Plasma drug concentration and time after injection of salicylic acid (O), *o*-cresotic acid (□), *m*-cresotic acid (△) and *p*-cresotic acid (+). Each point is mean of eight determinations.

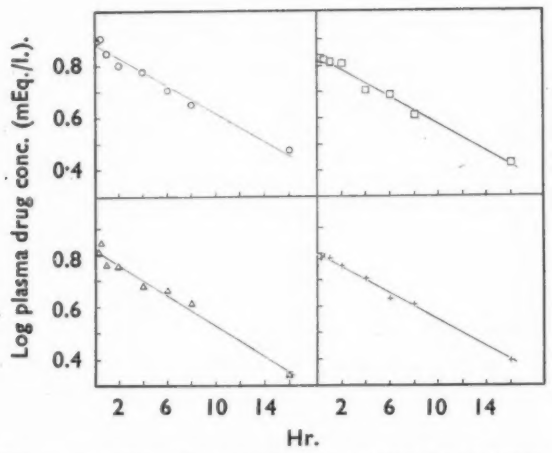

FIG. 3.—Log plasma drug concentration and time after injection of salicylic acid (O), *o*-cresotic acid (□), *m*-cresotic acid (△) and *p*-cresotic acid (+). Each point is mean of eight determinations.

cresotates might account for the difference in potencies was examined by comparing the rates of disappearance of the drugs from the blood after single intraperitoneal injections.

Rats which had received the previous maximum dose of salicylic acid (150 mg.) or a cresotic acid (100 mg.) were killed between 15 min. and 16 hr. later and the plasma drug concentration was determined. The mean plasma concentrations for each drug are shown in Fig. 2. In each case the plasma drug concentration showed an apparently exponential decline. Transformation of the concentrations to a logarithmic scale made the relationship rectilinear (Fig. 3).

The homogeneity of variance of the transformed observations was confirmed by Bartlett's test, which gave $0.50>P>0.10$; analyses of variance failed to show a significant deviation from rectilinear regression for any of the drugs (Table V). The regression lines were then formally compared.

TABLE V

ANALYSES OF VARIANCE OF LOG PLASMA DRUG CONCENTRATIONS AND REGRESSION EQUATIONS OF LOG PLASMA DRUG CONCENTRATION ON TIME AFTER INJECTION FOR SALICYLIC ACID AND THE THREE CRESOTIC ACIDS

x=time in hr.; y=log plasma drug concentration (m.Eq./l.).

Compound	Mean Squares			Regression Equation
	Due to Regression d.f.=1	Deviation from Regression d.f.=6	Residual d.f.=56	
Salicylic acid ..	1·1433	0·0047	0·0055	$y=0·89-0·027x$
o-Cresotic acid ..	1·0823	0·0027	0·0045	$y=0·84-0·026x$
m-Cresotic acid ..	1·3777	0·0070	0·0051	$y=0·82-0·029x$
p-Cresotic acid ..	1·0210	0·0058	0·0067	$y=0·80-0·025x$

TABLE VI

DISAPPEARANCE FROM BLOOD OF SALICYLATE AND CRESOTATES

Regressions of log plasma drug concentration on time after injection: analysis of covariance.

Source of Variance	Regression Coefficient	Deviation from Regression		
		Sum of Squares	d.f.	Variance
Salicylic acid	−0·0268	0·3342	62	0·00539
o-Cresotic acid	−0·0261	0·2674	62	0·00431
m-Cresotic acid	−0·0294	0·3299	62	0·00532
p-Cresotic acid	−0·0253	0·3787	62	0·00611
Within drugs		1·3102	248	0·00528
Regression coefficient ..		0·0151	3	0·00503
Common line	−0·0269	1·3253	251	0·00528
Elevation		0·2764	3	0·092
Total ..		1·6017	254	0·00631

Any rectilinear regression is fully defined in terms of two parameters, elevation and gradient. In the present experiments the elevations were mainly dependent on the initial dose of the drug injected; any difference in rates of disappearance of the drugs from the blood would, after logarithmic transformation of concentration, appear as a difference in gradient. The analysis of covariance which is summarized in Table VI confirmed that there was no significant difference between the four regression coefficients of log concentrations of the various drugs on time after injection.

There was thus no evidence that the rates of disappearance of the cresotates from blood differed among themselves or from that of salicylate.

DISCUSSION

In accordance with the practice in this laboratory the variability of biological material was accepted. No attempt was made to impose "basal" conditions; variations in exercise, feeding, etc., were assumed to be randomly distributed. The animals were, however, paired for sex and weight, and all the animals were chosen within an arbitrary weight range. Quality control charts showed that the experiments were in satisfactory statistical control.

The drugs which at the dose administered did not alter the rate of oxygen consumption of rats were 2:3-dihydroxybenzoic acid, phthalic acid, and 6-methylsalicylic acid.

Many of the compounds were tolerated at high doses. At such doses *m*- and *p*-hydroxybenzoic acid, 2:4-, 2:5-, 2:6-, 3:4-, and 3:5-dihydroxybenzoic acid depressed the rate of oxygen consumption of rats. 5-Aminosalicylic acid, salicylamide, salicyluric acid, and *o*-aminobenzoic acid were also metabolic depressants in doses similar to salicylic acid.

Gentisic acid (2:5-dihydroxybenzoic acid) and salicyluric acid have been isolated in the urine as metabolites of salicylic acid (Kapp and Coburn, 1942). Thus in the body some salicylic acid is converted from a metabolic stimulant to a depressant, which suggests that these two acids, salicyluric and gentisic, are true detoxication products in respect of their metabolic stimulant action.

It was already known that the antituberculous drug, *p*-aminosalicylic acid, depressed the rate of oxygen consumption in man (MacGregor and Somner, 1954) and it is interesting to note that the 5-aminosalicylic acid possessed a similar depressant property.

The only compounds which increased the rate of oxygen consumption of rats were salicylic acid and *o*-, *m*-, and *p*-cresotic acid.

These acids have many chemical, physical, and pharmacological properties in common. Chemically they are all phenolic acids with the hydroxyl group in the *ortho* position relative to the carboxyl group and they all give a purple colour with ferric chloride. May (1909) found that the cresotic acids resembled salicylic acid in their action as antifermentatives, as bactericides, as antipyretics, and as specifics in acute rheumatism. Stockman (1912) used the sodium salts of the three cresotic acids in the treatment of rheumatic fever and concluded that for practical purposes they were inferior to sodium salicylate.

Sodium gentisate (Meyer and Ragan, 1948) and sodium γ-resorcylate (sodium 2:6-dihydroxybenzoate) (Reid, Watson, Cochran, and Sproull, 1951) have been reported as effective in the treatment of acute rheumatism, but neither increases the rate of oxygen consumption of rats. These results indicate that metabolic stimulant action in rats is not essential for therapeutic activity, although, within this series, the ability to increase the rate of oxygen consumption may be sufficient for anti-rheumatic activity.

The dose/response curves of the three cresotic acids and salicylic acid were rectilinear. There was therefore no need to transform dose to a logarithmic scale. The present dose/response relationship is consistent with the results of Alexander and Johnson (1956) on man and of Reid (1957) on rabbits. The comparative assay of the four metabolic stimulants described in the present investigation was novel both because the common intercept on the *y*-axis was negative and because it was a comparative assay, and therefore better not treated in terms of multiple regression. A procedure devised by Silvey (personal communication) was therefore used.

The cresotic acids were found to be more powerful metabolic stimulants than salicylic acid when administered in single doses intraperitoneally to rats; *o*-cresotic acid was the most powerful with a potency ratio relative to salicylate of 2.61, *m*-cresotic acid had a ratio of 1.78, and *p*-cresotic acid a ratio of 1.89. The fiducial limits of the ratios showed that these differences were significant at the 95% level. They were calculated on a molar basis, and would have been numerically lower, but equally significant, if computed weight for weight. Such a basis would have taken no account of the chemical equivalence of the drugs.

The introduction of the methyl group into the benzene ring therefore increased the potency of salicylic acid as a metabolic stimulant; the *ortho* position was the most effective. This raises the question of the effect of other alkyl or aryl substituents in the *ortho* position. It is of interest to note that preliminary observations with 3-phenylsalicylic acid suggest that it is even more potent than *o*-cresotic acid.

The present results establish that these differences in the potencies of the cresotic acids relative to salicylate are not dependent on variations in the rates of degradation or excretion of the drugs nor on differences in the primary actions. It is concluded that the tissues themselves might therefore be more sensitive to the cresotates than salicylate.

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DERIVATIVES OF 3 : 4-XYLIDINE AND RELATED COMPOUNDS AS INHIBITORS OF INFLUENZA VIRUS : RELATIONSHIPS BETWEEN CHEMICAL STRUCTURE AND BIOLOGICAL ACTIVITY

BY

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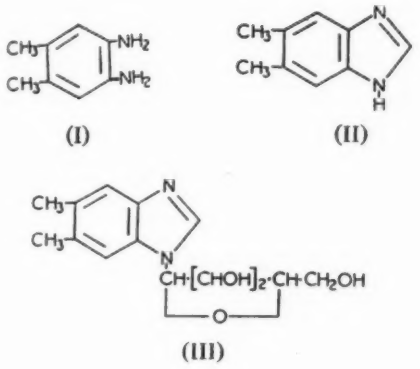
A series of compounds, based primarily on 3 : 4-xylidine, was examined for inhibitory activity towards the growth of influenza virus in tissue culture. Marked dependence of inhibitory activity upon chemical structure was observed particularly when the 3 : 4-xylyl group was replaced by other simple aryl radicals. *N*-(2-Piperidinoethyl)-3 : 4-xylidine dihydrochloride, a typical compound combining high intrinsic inhibitory activity with no obvious toxicity towards the host tissues, did not inactivate the virus directly before its adsorption, did not interfere with adsorption of virus by the tissues, and did not inhibit the release of freshly synthesized virus by the tissues, but specifically depressed the synthesis of viral haemagglutinin to a greater extent than it depressed the synthesis of complement-fixing soluble antigen. The inhibition of growth of influenza virus caused by this compound in tissue culture was reversed by appropriate addition of 4 : 5-dimethyl-*o*-phenylenediamine, but not apparently by riboflavin or by vitamin B_{12}. The action of this substance, and, by inference, of related compounds, in inhibiting viral synthesis may be the result of depressed cytoplasmic protein synthesis.

The effective chemotherapy of virus infections must combine inhibition of growth of the virus and lack of toxicity to the host, and the intimate dependence of the virus upon the metabolism of the susceptible host cell introduces difficulties which are absent from the chemotherapy of infections due to free-living bacterial and protozoal pathogens which possess active and unique metabolic patterns of their own. Both qualitative and quantitative differences may, however, exist between the biosynthetic requirements of virus and host cell, and these differences, if they can be detected, may allow the selective inhibition of virus multiplication by appropriate compounds. Although numerous substances are now known to influence the growth of influenza virus in tissue cultures, few definite pointers towards the effective chemotherapy of influenza have emerged. Structure/activity relationships, however, are more readily detectable in such cultures which are capable of providing useful information concerning the metabolic requirements for viral growth, or conditions for viral inhibition, in a cellular environment. The present communication describes a series of compounds which appeared to affect the growth of influenza virus in tissue culture in doses which were almost always without a gross toxic effect on the host tissue.

We decided to examine *N*-acyl derivatives of 4 : 5-dimethyl-*o*-phenylenediamine (I) bearing in the acyl radical basic groups capable of conferring solubility in water on the resulting compounds through the formation of neutral soluble salts as there were indications that this amine and related compounds had some significant biological activities in addition to its incorporation into riboflavin and into vitamin B_{12}. For example, it had been shown that the acid degradation product of vitamin B_{12}, 5 : 6-dimethylbenzimidazole (II) and its precursor (I) were without vitamin activity for exacting lactobacilli and were in fact toxic to these organisms (Hendlin and Soars, 1951), although 4 : 5-dichloro-*o*-phenylenediamine was an inhibitor of growth of various bacteria and the inhibitory effects were reversed by (I) (Woolley and Pringle, 1952). It was also shown (Tamm, Folkers, Shunk, and Horsfall, 1954) that 5 : 6-dimethylbenzimidazole (II) was inhibitory towards influenza virus although 5 : 6-dimethyl-1-α-D-ribofuranosylbenzimidazole (III) (α-ribazole), a moiety incorporating (II) present in vitamin B_{12}, was not inhibitory under similar circumstances. Replace-



(I) (II)

(III)

ment of the two methyl groups in (III) by chlorine atoms, however, gave a very active inhibitor of the growth of influenza virus (Tamm *et al.*, 1954), and a further increase in activity was found in 4:5:6(or 5:6:7)-trichloro-1-β-D-ribofuranosylbenzimidazole (Tamm, Folkers, and Shunk, 1956).

METHODS

Chemical Methods

General Methods for the Preparation of Halogenoacylarylamides

Two methods were used: (i) the appropriate amine and chloroacetyl (or α-chloropropionyl) chloride were heated together in boiling benzene for 2 hr., or (ii) the amine and chloroacetyl chloride were allowed to react in glacial acetic acid in the cold in presence of sodium acetate as described by Löfgren (1948).

The following compounds have not been described previously (the yield and method of preparation follow the name of the compound): *α-chloro-6-nitroaceto-3:4-xylidide*, 88%, (i), yellow needles from benzene, m.p. 150° (Found: C, 49.6; H, 4.7. $C_{10}H_{11}O_3N_2Cl$ requires C, 49.5; H, 4.6%); *α-chloro-3:4-dimethoxyacetanilide*, 81%, (i), colourless needles from aqueous ethanol, m.p. 133 to 135° (Found: C, 52.2; H, 5.1. $C_{10}H_{12}O_3NCl$ requires C, 52.3; H, 5.2%); *α:3:4-trichloroacetanilide*, 89%, (ii), colourless needles from aqueous ethanol, m.p. 105 to 107° (Found: C, 40.5; H, 2.5; N, 6.1. $C_8H_6ONCl_3$ requires C, 40.3; H, 2.5; N, 5.9%); *α-chloro-N-diphenyl-2-ylacetamide*, 92%, (ii), colourless rods from aqueous ethanol, m.p. 98 to 100° (Found: C, 68.4; H, 4.9. $C_{14}H_{12}ONCl$ requires C, 68.4; H, 4.9%); *α-chloro-N-diphenyl-4-ylacetamide*, 80%, (ii), colourless plates from methanol, m.p. 176 to 178° (Found: C, 68.5; H, 5.0%); *α:5-dichloroaceto-o-toluidide*, 82%, (ii), colourless plates from aqueous ethanol, m.p. 128 to 130° (Found: C, 49.5; H, 3.9. $C_9H_9ONCl_2$ requires C, 49.6; H, 4.2%); *α-chloropropiono-3:4-xylidide*, 88%, (i), colourless needles from aqueous ethanol, m.p. 139 to 140° (Found: C, 62.4; H, 6.4. $C_{11}H_{14}ONCl$ requires C, 62.4; H, 6.7%).

Table I gives the explanation of symbols used in subsequent Tables.

TABLE I

EXPLANATION OF SYMBOLS USED IN SUBSEQUENT TABLES TO SHOW EXTENT OF INHIBITORY ACTIVITY

Mean Log_2 Control Titre −Mean Log_2 Test Titre	Yield of Virus as % of Control	Inhibitory Activity
<1·0	⩾ 50	−
1·0–2·0	25–50	+
2·0–3·0	12·5–25	++
3·0–4·0	6·25–12·5	+++
>4·0	<6·25	++++

General Methods for the Preparation of Piperidino-, Pyrrolidin-1-yl, and Dialkylamino-acylarylamides and their Hydrochlorides

The appropriate halogenoacylarylamide was heated with 2 molecular proportions of the requisite amine in benzene for 5 hr. Precipitated amine hydrochloride was collected and the filtrate was taken to dryness. Basic material in the residue was recovered by solution in 3 N-HCl, filtration, and ether extraction, followed by basification with ammonia. The resulting free base was then either collected or extracted with ether. The hydrochlorides were prepared by passing hydrogen chloride into acetone solutions of the bases. Salts which separated were collected and more soluble ones were isolated by evaporation of the acetone; they were then recrystallized either from methanol/ethyl acetate or from ethanol/ether (Table II).

6-Amino-α-piperidinoaceto-3:4-xylidide (IV) *Dihydrochloride.* — 6-Nitro-α-piperidinoaceto-3:4-xylidide (93% yield) separated from *n*-propanol in yellow needles, m.p. 272 to 273° (Found: C, 62.1; H, 7.4. $C_{15}H_{21}O_3N_3$ requires C, 61.9; H, 7.3%). 8.1 g. of the substance was reduced in ethanol (200 ml.) in the presence of Raney nickel in hydrogen at room temperature and 3 atm.; the product was isolated in the usual way and converted into the dihydrochloride (Table II, 14).

α-Piperazin-1′-ylaceto-3:4-xylidide Hydrochloride. —α-Chloroaceto-3:4-xylidide (3.94 g.) and benzyloxycarbonylpiperazine (8.8 g.) were allowed to react in benzene in the manner described above. The intermediate α-(benzyloxycarbonylpiperazin-1-yl)-aceto-3:4-xylidide was a crystalline solid (5.35 g.), m.p. 177 to 180°. Removal of the benzyloxycarbonyl group was effected in ethanol solution at room temperature and atmospheric pressure in hydrogen in the presence of 5% palladized charcoal (14 hr.) (Table II, 28).

Glycyl-3:4-xylidide. — α-Chloroaceto-3:4-xylidide (19.8 g.) was allowed to react with saturated (0°) ethanolic ammonia (500 ml.), following the method described by Hill and Kelsey (1922) for the 4-chloro analogue, affording secondary amine (3.18 g.), m.p. 164 to 166°, and a main crop (13.4 g.) of the required primary amine (Table II, 32).

TABLE II

INHIBITORY ACTIVITY AND CERTAIN PHYSICAL AND CHEMICAL PROPERTIES OF PIPERIDINO-, PYRROLIDIN-1-YL, AND DIALKYLAMINO-ACYLARYLAMIDES AND THEIR HYDROCHLORIDES

Aryl·NH·CO·CHR·NR^1R^2

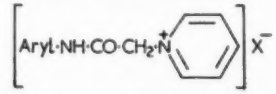
Symbols as in Table I. a=aqueous ethanol; b=ethanol/ether; c=methanol/ethyl acetate; d=ethyl acetate/petrol; e=benzene; f=benzene/petrol; g=needles; h=plates; k=Erdtman and Löfgren (1937) record base, m.p. 99 to 100°; l=Löfgren (1946) records base, m.p. 96 to 98°, hydrochloride, m.p. 171 to 172°; m=Löfgren and Lundquist (1946) record base, m.p. 115 to 116°, hydrochloride, m.p. 177 to 180°; n=Hall and Turner (1948); o=Löfgren (1946) records hydrochloride, m.p. 168 to 169°; t=toxic.

		Inhibitory Activity (0·1 mg./ml.)	Yield (%)	M.p. (Solvent; Cryst. Form)	Mol. Formula	Found (%)			Required (%)		
						C	H	N	C	H	N
1	α-Piperidinoacetanilide[k]		54	97–98° (ag)	$C_{13}H_{18}ON_2$	71·7	8·2	12·6	71·5	8·3	12·8
	hydrochloride	+	—	181–183° (hg)	$C_{13}H_{18}ON_2$,HCl	61·4	7·6	11·1	61·3	7·5	11·0
2	α-Piperidinoacetbenzylamide		—	43–46°							
	hydrochloride	++	66	148–150° (ch)	$C_{14}H_{20}ON_2$,HCl	62·7	7·6	10·1	62·5	7·9	10·4
3	α-Piperidinoaceto-*o*-toluidide[l]		77	95–98° (ah)	$C_{14}H_{20}ON_2$	72·5	8·7	11·9	72·4	8·7	12·1
	hydrochloride	−	—	168–171°							
4	α-Piperidinoaceto-*m*-toluidide		—								
	hydrochloride	+++	65	185–187° (cg)	$C_{14}H_{20}ON_2$,HCl	62·6	7·6	10·4	62·5	7·9	10·4
5	α-*Piperidinoaceto*-p-*toluidide*		85	65–67° (ag)	$C_{14}H_{20}ON_2$	72·3	8·4	11·9	72·4	8·7	12·1
	hydrochloride	++	—	212–214°							
6	α-Piperidinoaceto-2: 3-xylidide		84	73–75° (ag)							
	hydrochloride	+	—	221–223° (cg)	$C_{15}H_{22}ON_2$,HCl	63·6	8·0	10·0	63·7	8·2	9·9
7	α-*Piperidinoaceto*-2: 4-*xylidide*		77	86–88° (ag)	$C_{15}H_{22}ON_2$	73·0	9·0	11·2	73·2	9·0	11·4
	hydrochloride	−	—	217–219°							
8	α-*Piperidinoaceto*-2: 5-*xylidide*		83	95–96° (ah)	$C_{15}H_{22}ON_2$	73·0	8·7	11·4	73·2	9·0	11·4
	hydrochloride	++	—	185–187°							
9	α-Piperidinoaceto-2: 6-xylidide[m]		79	112–114° (ag)	$C_{15}H_{22}ON_2$	73·0	8·9	11·3	73·2	9·0	11·4
	hydrochloride	−	—	183–185°							
10	α-*Piperidinoaceto*-3: 4-*xylidide* (V)		85	71–73° (ag)	$C_{15}H_{22}ON_2$	73·0	8·9	11·4	73·2	9·0	11·4
	hydrochloride	+++	—	189–191°							
11	α-*Piperidinoaceto*-3: 5-*xylidide*		72	87–89° (ag)	$C_{15}H_{22}ON_2$	73·2	8·9	11·6	73·2	9·0	11·4
	hydrochloride	−	—	216–218°							
12	2: 4: 5-Trimethyl-α-piperidinoacetanilide		—								
	hydrochloride	+	84	246–248° (cg)	$C_{16}H_{24}ON_2$,HCl	64·6	8·5	9·2	64·7	8·5	9·4
13	2: 4: 6-*Trimethyl-α piperidinoacetanilide*		94	104–106° (ah)	$C_{16}H_{24}ON_2$	74·2	9·2	10·8	73·8	9·3	10·8
	hydrochloride	+	—	195–196° (bg)							
14	6-Amino-α-piperidinoaceto-3: 4-xylidide (IV)		—								
	dihydrochloride	−	72	272–275° (ch)	$C_{15}H_{22}ON_2$,2HCl	53·9	7·4	12·3	53·9	7·5	12·6
15	3: 4-*Dimethoxy-α-piperidinoacetanilide*		88	78–79° (ah)	$C_{15}H_{22}O_3N_2$	64·6	7·7	10·0	64·7	8·0	10·0
	hydrochloride	−	—	183–185°							
16	α-*Piperidino*-N-*diphenyl*-2-*ylacetamide*		92	134–135° (ag)	$C_{19}H_{22}ON_2$	77·6	7·4	9·4	77·5	7·2	9·5
	hydrochloride	pptd	—	169–170°							
17	α-*Piperidino*-N-*diphenyl*-4-*ylacetamide*		93	108–110° (ag)	$C_{19}H_{22}ON_2$	77·6	7·6	9·7	77·5	7·2	9·5
	hydrochloride	pptd	—								
18	p-*Chloro-α-piperidinoacetanilide*		85	86–88° (ag)	$C_{13}H_{17}ON_2Cl$	61·6	6·8	10·8	61·8	6·8	11·1
	hydrochloride	+	—	220–222°							
19	3: 4-*Dichloro-α-piperidinoacetanilide*		58	74–76° (ag)	$C_{13}H_{16}ON_2Cl_2$	54·4	5·6	9·7	54·4	5·6	9·8
	hydrochloride	++++[t]	—	196–198°							
20	5-Chloro-α-piperidinoaceto-*o*-toluidide		—								
	hydrochloride	++	87	235–237° (ch)	$C_{14}H_{19}ON_2Cl$,HCl	55·2	6·5	9·3	55·5	6·7	9·0
21	p-*Bromo-α-piperidinoacetanilide*		67	85–88° (ag)	$C_{13}H_{17}ON_2Br$	52·4	5·6	9·7	52·2	5·8	9·4
	hydrochloride	++++	—	229–231°							
22	*p*-Iodo-α-piperidinoacetanilide		—								
	hydrochloride	++++	87	218–220° (cg)	$C_{13}H_{17}ON_2I$,HCl	40·7	5·0	7·5	41·0	4·8	7·4
23	4-Chloro-2-nitro-α-piperidinoacetanilide[n]		—								
	hydrochloride	pptd	—	230–232° (bg)	$C_{13}H_{16}O_3N_3Cl$,HCl	46·5	5·3	12·7	46·7	5·1	12·6
24	2-Amino-4-chloro-α-piperidinoacetanilide										
	hydrochloride[n]	+[t]									
25	α-Piperidino-*p*-sulphamoylacetanilide		90	196–198°							
	hydrochloride	−	—	268–270° (cg)	$C_{13}H_{19}O_3N_3S$,HCl	47·0	5·8	12·4	46·8	6·0	12·7
26	α-Pyrrolidin-1′-ylaceto-3: 4-xylidide		—								
	hydrochloride	++++	78	200–203° (cg)	$C_{14}H_{20}ON_2$,HCl	62·2	7·7	10·5	62·6	7·9	10·5
27	α-*Morpholinoaceto*-3: 4-*xylidide*		52	83–85° (ah)	$C_{14}H_{20}O_2N_2$	67·7	8·3	11·5	67·7	8·1	11·3
	hydrochloride	−	—	222–224°							
28	α-Piperazin-1′-ylaceto-3: 4-xylidide		—								
	hydrochloride	++++	31	242–245° (cg)	$C_{14}H_{21}ON_3$,HCl	59·0	7·6	14·4	59·2	7·8	14·8
29	α-*Piperidinopropiono*-3: 4-*xylidide*		72	78–80° (ag)	$C_{16}H_{24}ON_2$	74·0	9·1	11·0	73·8	9·2	10·8
	hydrochloride	++++	—	178–181°							
30	α-Pyrrolidin-1′-ylpropiono-3: 4-xylidide		78	225–227° (ch)	$C_{16}H_{22}ON_2$,HCl	63·9	8·2	9·7	63·8	8·2	9·9

27	α-*Morpholinoaceto*-3: 4-*xylidide* hydrochloride	++++	78 52	200–203° (cg) 83–85° (ah)	$C_{14}H_{20}ON_2$,HCl $C_{14}H_{20}O_2N_2$	62·2 67·7	7·7 8·3	10·5 11·5	62·6 67·7	7·9 8·1	10·5 11·3
28	α-Piperazin-1′-ylaceto-3: 4-xylidide hydrochloride	−	—	222–224°							
29	α-*Piperidinopropiono*-3: 4-*xylidide* hydrochloride	++++ ++++	31 72	242–245° (cg) 78–80° (ag) 178–181°	$C_{14}H_{21}ON_2$,HCl $C_{16}H_{24}ON_2$	59·0 74·0	7·6 9·1	14·4 11·0	59·2 73·8	7·8 9·2	14·8 10·8
30	α-Pyrrolidin-1′-ylpropiono-3: 4-xylidide hydrochloride	++++	78	225–227° (ch)	$C_{16}H_{22}ON_2$,HCl	63·9	8·2	9·7	63·8	8·2	9·9
31	3: 4-Dimethoxy-α-piperidinopropionanilide hydrochloride	−	78	197–199° (ch)	$C_{16}H_{24}O_3N_2$,HCl	58·2	7·5	8·7	58·4	7·7	8·5
32	*Glycyl*-3: 4-*xylidide* hydrochloride	− −	87 —	97–99° (ag) 259–261° (cg)	$C_{10}H_{14}ON_2$ $C_{10}H_{14}ON_2$,HCl	67·3 55·9	8·0 7·0	15·3 13·0	67·4 55·9	7·9 7·0	15·7 13·1
33	α-Dimethylaminoaceto-3: 4-xylidide hydrochloride	−	48	166–168° (ch)	$C_{12}H_{18}ON_2$,HCl	59·6	7·8	11·8	59·4	7·9	11·6
34	α-Diethylaminoaceto-3: 4-xylidide hydrochloride[o]	+++	72	165–167° (ch)	$C_{14}H_{22}ON_2$,HCl	62·3	8·2	10·5	62·1	8·2	10·3
35	α-Dipropylaminoaceto-3: 4-xylidide hydrochloride	++	82	154–156° (dg)	$C_{16}H_{26}ON_2$,HCl	64·5	8·9	9·2	64·3	9·1	9·4
36	α-Dibutylaminoaceto-3: 4-xylidide hydrochloride	pptd	31	155–157° (fg)	$C_{18}H_{30}ON_2$,HCl	66·1	9·6	8·3	66·1	9·6	8·6
37	α-Diallylaminoaceto-3: 4-xylidide hydrochloride	++++	63	133–136° (eh)	$C_{16}H_{22}ON_2$,HCl	64·9	7·8	9·5	65·2	7·9	9·5

TABLE III

INHIBITORY ACTIVITY AND CERTAIN PHYSICAL AND CHEMICAL PROPERTIES OF 1-ARYLCARBAMOYLMETHYLPYRIDINIUM CHLORIDES AND OTHER QUATERNARY AMMONIUM SALTS

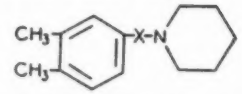


and others.

Symbols as in Table I. a–h, as in Table II; p = Mel'nikov, Sukhareva and Arkhipova (1948) record m.p. 182°; q = Mel'nikov *et al.* (1948) record m.p. 220°; r = yellow needles from dimethylformamide/ether; s = slightly toxic.

		Inhibitory Activity (0·1 mg./ml.)	Yield (%)	M.p. (Solvent, Cryst. Form)	Mol. Formula	Found (%)			Required (%)		
						C	H	N	C	H	N
38	1-*o*-Tolylcarbamoylmethylpyridinium chloride[p]	−	66	190–192° (cg)	$C_{14}H_{15}ON_2Cl$	64·3	5·8	10·4	64·0	5·8	10·7
39	1-m-*Tolylcarbamoylmethylpyridinium chloride*	−	76	218–220° (ch)	$C_{14}H_{15}ON_2Cl$	64·2	5·9	10·2	64·0	5·8	10·7
40	1-*p*-Tolylcarbamoylmethylpyridinium chloride[q]	+	67	245–247° (ch)	$C_{14}H_{15}ON_2Cl$	64·4	5·6	10·5	64·0	5·8	10·7
41	1-(2 : 3-*Xylylcarbamoylmethyl*)*pyridinium chloride*	−	43	197–199° (cg)	$C_{15}H_{17}ON_2Cl$	65·1	6·1	10·2	65·1	6·2	10·1
42	1-(2 : 4-*Xylylcarbamoylmethyl*)*pyridinium chloride*	+	52	165–167° (cg)	$C_{15}H_{17}ON_2Cl$	65·0	6·3	10·1	65·1	6·2	10·1
43	1-(2 : 5-*Xylylcarbamoylmethyl*)*pyridinium chloride*	−	64	184–186° (cg)	$C_{15}H_{17}ON_2Cl$	65·3	6·0	9·9	65·1	6·2	10·1
44	1-(2 : 6-*Xylylcarbamoylmethyl*)*pyridinium chloride*	−	50	197–199° (cg)	$C_{15}H_{17}ON_2Cl$	64·7	6·3	10·3	65·1	6·2	10·1
45	1-(3 : 4-*Xylylcarbamoylmethyl*)*pyridinium chloride* (VI)	++++	75	236–238° (cg)	$C_{15}H_{17}ON_2Cl$	64·6	6·2	10·0	65·1	6·2	10 1
46	1-(3 : 5-*Xylylcarbamoylmethyl*)*pyridinium chloride*	−	35	164–166° (ch)	$C_{15}H_{17}ON_2Cl$	65·3	6·3	9·9	65·1	6·2	10·1
47	1-(2 : 4 : 5-*Mesitylcarbamoylmethyl*)*pyridinium chloride*	−	81	190–192° (cg)	$C_{16}H_{19}ON_2Cl$	66·1	6·5	9·6	66·1	6·6	9·6
48	1-(3 : 4-*Dimethoxyphenylcarbamoylmethyl*)*pyridinium chloride*	−	60	201–203° (cg)	$C_{15}H_{17}O_3N_2Cl$	58·2	5·3	9·1	58·3	5·6	9·0
49	1-(*Diphenyl*-4-*ylcarbamoylmethyl*)*pyridinium chloride*	++++[t]	86	242–244° (cg)	$C_{19}H_{17}ON_2Cl$	69·8	5·5	8·4	70·1	5·3	8·7
50	1-m-*Chlorophenylcarbamoylmethylpyridinium chloride*	+++	76	256–258° (cg)	$C_{13}H_{12}ON_2Cl_2$	55·3	4·3	10·0	55·1	4·3	9·9
51	1-p-*Chlorophenylcarbamoylmethylpyridinium chloride*	+	62	230–232° (cg)	$C_{13}H_{12}ON_2Cl_2$	55·1	4·1	9·8	55·1	4·3	9·9
52	1-(3 : 4-*Dichlorophenylcarbamoylmethyl*)*pyridinium chloride*	++++[s]	62	282–284° (cg)	$C_{13}H_{11}ON_2Cl_3$	49·5	3·7	8·8	49·1	3·5	8·8
53	1-(5-*Chloro*-o-*tolylcarbamoylmethyl*)*pyridinium chloride*	+++	49	214–216° (cg)	$C_{14}H_{14}ON_2Cl_2$	56·2	4·7	9·1	56·6	4·8	9·4
54	1-p-*Bromophenylcarbamoylmethylpyridinium chloride*	+++	61	235–237° (cg)	$C_{13}H_{12}ON_2BrCl$	47·9	3·6	8·4	47·7	3·7	8·6
55	1-*p*-Iodophenylcarbamoylmethylpyridinium chloride *picrate*	++++	71	248–250° (ch) 216–218° (r)	$C_{19}H_{14}O_8N_5I$	40·4	2·9	12·1	40·1	2·8	12·3
56	4-*Methyl*-1-(3 : 4-*xylylcarbamoylmethyl*)*pyridinium chloride*	+++	68	219–222° (ch)	$C_{16}H_{19}ON_2Cl$	65·9	6·5	9·6	66·1	6·6	9·6
57	3-*Carbamoyl*-1-(3 : 4-*xylylcarbamoylmethyl*)*pyridinium chloride*	−	86	275–277° (ag)	$C_{16}H_{18}O_2N_2Cl$	60·2	5·7	13·3	60·1	5·7	13·1
58	1-*Methyl*-1-(3 : 4-*xylylcarbamoylmethyl*)*piperidinium iodide*	−	70	177–180° (ch)	$C_{16}H_{25}ON_2I$	49·3	6·4	7·0	49·5	6·5	7·2
59	1-(3 : 4-*Dimethoxyphenylcarbamoylmethyl*)-1-*methylpiperidinium iodide*	−	85	176° (ch)	$C_{16}H_{25}O_3N_2I$	45·8	6·0	6·4	45·7	6·0	6·7
60	*Triallyl*(3 : 4-*xylylcarbamoylmethyl*)*ammonium bromide*	+	59	150–152° (bg)	$C_{19}H_{27}ON_2Br$	60·4	7·3	7·4	60·2	7·2	7·4
61	1-(2-*Amino*-4-*chlorophenylcarbamoylmethyl*)-1-*methylpiperidinium iodide*	−	71	205–207° (ag)	$C_{14}H_{21}ON_3ClI$	41·1	5·2	10·0	41·0	5·2	10·2

TABLE IV
MISCELLANEOUS COMPOUNDS

Other compounds of the type  and others.

s = slightly toxic; t = toxic; u = King and Tonkin (1946); v = Walker (1940).

		X	Inhibitory Activity (0·1 mg./ml.)	Mol. Formula	Found (%)			Required (%)		
					C	H	N	C	H	N
62	*N*-(2-Piperidinoethyl)-3: 4-xylidine *dihydrochloride* (VII)	$NH.CH_2.CH_2$	++++	$C_{15}H_{24}N_2,2HCl$	59·1	8·7	9·4	59·0	8·6	9·2
63	*N*-(3-Piperidinopropyl)-3: 4-xylidine *hydrochloride*	$NH.CH_2.CH_2.CH_2$	++++	$C_{16}H_{26}N_2,HCl$	67·8	9·6	9·9	67·9	9·6	10·0
64	*N*-(2-Piperidinopropyl)-3: 4-xylidine *dihydrochloride*	$NH.CH_2.CH(CH_3)$	+++++[t]	$C_{16}H_{26}N_2.2HCl$	59·8	8·7	8·6	60·2	8·8	8·8
65	*N*-2-(3: 4-Xylyloxy)ethylpiperidine *hydrochloride*	$O.CH_2.CH_2$	+++++[?s]	$C_{15}H_{23}ON,HCl$	66·8	8·6	5·2	66·8	8·6	5·2
66	*N*-3-(3: 4-Xylyloxy)propylpiperidine *hydrochloride*	$O.CH_2.CH_2.CH_2$	+++++[?s]	$C_{16}H_{25}ON,HCl$	67·4	8·9	4·7	67·7	9·2	4·9
67	1-*Piperidino*-3-(3: 4-*xylyloxy*)*propan*-2-*ol* *hydrochloride*	$O.CH_2.CH(OH).CH_2$	++++	$C_{16}H_{25}O_2N$ $C_{16}H_{25}O_2N,HCl$	72·8 64·3	9·5 9·0	5·5 5·0	73·0 64·1	9·5 8·7	5·3 4·7
68	*N*-2-(3: 4-Xylyl)ethylpiperidine *hydrochloride*	$CH_2.CH_2$	+++++[?s]	$C_{15}H_{23}N,HCl$	70·7	9·6	5·5	71·0	9·5	5·5
69	*N*-3-(3: 4-Xylyl)propylpiperidine *hydrochloride*	$CH_2.CH_2.CH_2$	+++++[s]	$C_{16}H_{25}N,HCl$	72·0	9·7	5·2	71·8	9·8	5·2
70	*N*-4-(3: 4-Xylyl)butylpiperidine *hydrochloride*	$CH_2.CH_2.CH_2.CH_2$	+++++[t]	$C_{17}H_{27}N,HCl$	72·2	9·7	4·9	72·5	10·0	5·0
71	Piperidinomethyl 3: 4-xylyl ketone *hydrochloride*	$CO.CH_2$	++	$C_{15}H_{21}ON,HCl$	67·0	8·2	5·5	67·3	8·3	5·2
72	2-Piperidinoethyl 3′: 4′-xylyl ketone *hydrochloride*	$CO.CH_2.CH_2$	++++[t]	$C_{16}H_{23}ON,HCl$	68·3	8·3	4·9	68·2	8·6	5·0
73	(*N*-α-Piperidinoacetylglycyl)-3: 4-xylidide *hydrochloride*	$NH.CO.CH_2.NH.CO.CH_2$	+	$C_{17}H_{25}ON_3,HCl$	59·6	7·7	12·2	59·9	8·0	12·3
74	N^1N^1-cyclo*Pentamethylene*-N^5-3: 4-*xylyldiguanide* hydrochloride	$NH.C(:NH).NH.C(:NH)$	++++[t]	$C_{15}H_{23}N_5$	65·9	8·4	25·7	65·9	8·5	25·6
75	*N*-(2-Pyrrolidin-1′-ylethyl)-3: 4-xylidine *dihydrochloride*		++++	$C_{14}H_{22}N_2.2HCl$	58·0	8·5	9·7	57·7	8·3	9·6
76	N^1N^1-*cyclo*Tetramethylene-N^5-3: 4-xylyldiguanide *hydrochloride*		++++[t]	$C_{14}H_{21}N_5,HCl$	56·8	7·3	23·9	56·8	7·5	23·7
77	N^1-*m*-Tolyldiguanide hydrochloride[u]		−							
78	N^1-*p*-Tolyldiguanide hydrochloride[u]		−							
79	N^1-2: 5-Xylyldiguanide hydrochloride[u]		++							
80	N^1-3: 4-Xylyldiguanide hydrochloride[u]		++++[s]							
81	3: 4-Xylylguanidine nitrate[u]		+++++[t]							
82	3: 4-Dimethylbenzamidine *hydrochloride*		+++++[t]	$C_9H_{12}N_2,HCl$	58·6	7·1	14·8	58·6	7·1	15·2
83	*N′N′*-Diethyl-N^5-(2-diethylaminoethyl)sulphanilamide hydrochloride[v]		++							
84	*N′*-2-Diethylaminoethyl-*N′N′-cyclo*pentamethylene-sulphanilamide hydrochloride[v]		++++							
85	4: 5-Dimethyl-2-(3-piperidinopropylamino)thiazole *dihydrochloride*		+	$C_{13}H_{23}N_3S,2HCl$	48·0	7·6	12·9	47·8	7·7	12·9

(N-α-*Piperidinoacetylglycyl*)-3:4-*xylidide*. — (i) The preceding base (3.6 g.) was treated with chloroacetyl chloride in glacial acetic acid in the usual manner and the resulting (N-α-*chloroacetylglycyl*)-3:4-*xylidide* crystallized from aqueous ethanol in colourless needles (2.9 g.), m.p. 190 to 192° (Found: C, 56.6; H, 5.8; N, 11.0. $C_{12}H_{15}O_2N_2Cl$ requires C, 56.3; H, 5.6; N, 11.2%). (ii) Condensation (of 1.3 g.) with piperidine (0.85 g.) in benzene afforded (*N*-α-piperidinoacetylglycyl)-3:4-xylidide (0.22 g.), m.p. 198 to 200°, and the hydrochloride separated from methanol/ethyl acetate in colourless needles, m.p. 237 to 239° (Table IV, 73).

General Method for the Preparation of 1-*Arylcarbamoylmethylpyridinium Chlorides*

The appropriate halogenoacylarylamide (1 mol.) and pyridine (1.1 mol.) were allowed to react in boiling ethanol for 4 hr. Alcohol was then removed and the products were recrystallized from methanol/ethyl acetate. The picoline (Table III, 56) and nicotinamide (Table III, 57) derivatives were similarly prepared.

The remaining quaternary ammonium salts (Nos. 58 to 61) were obtained from the appropriate tertiary amine (Table II, 11, 15, 24) and methyl iodide in hot methanol (2 hr.), or by heating the tertiary base (Table II, 37) with allyl bromide in a sealed tube at 100° for 5 hr. (Table III, 60).

N-(2-*Piperidinoethyl*)-3:4-*xylidine Dihydrochloride* (VII).—A solution of α-piperidinoaceto-3:4-xylidide (2.46 g.) in tetrahydrofuran (20 ml.) was added dropwise with stirring to crushed lithium aluminium hydride (0.76 g.) in tetrahydrofuran (30 ml.) and the mixture was boiled under reflux for 4 hr. Excess lithium aluminium hydride was destroyed by the addition of 95% ethanol, followed by water and ether, and stirring was continued for 30 min. After filtration the organic phase was separated, dried, and evaporated. A solution of the residual gum in dry acetone (20 ml.) was saturated with dry hydrogen chloride, and recrystallization of the solid, which separated, from methanol/ethyl acetate afforded colourless needles (2.30 g.) of the dihydrochloride, m.p. 176 to 178° (Table IV, 62).

In similar fashion, α-pyrrolidin-1′-ylaceto-3:4-xylidide afforded *N*-(2-pyrrolidin-1′-ylethyl)-3:4-xylidine dihydrochloride, which separated from ethanol/ether in plates (21%), m.p. 172 to 176° (Table IV, 75), and α-piperidinopropiono-3:4-xylidide, reduced for 8 hr. in di-*n*-butyl ether, gave *N*-(2-piperidinopropyl)-3:4-xylidine dihydrochloride, crystallizing from methanol/ethyl acetate in plates (64%), m.p. 180 to 182° (Table IV, 64).

N-(3-*Piperidinopropyl*)-3:4-*xylidine Hydrochloride*. —A mixture of 3:4-xylidine (12.1 g.) and 3-piperidinopropyl chloride (Marxer, 1941) (17.8 g.) in ethanol (60 ml.) was boiled under reflux for 60 hr. During this time a crystalline solid separated. The solid was dissolved by the addition of more ethanol (charcoal) and, on cooling, *N*-(3-piperidinopropyl)-3:4-xylidine hydrochloride separated in colourless needles (18 g.), m.p. 222 to 224° (Table IV, 63).

2-(3:4-*Xylyloxy*)*ethyl Chloride*.—(i) A mixture of 3:4-xylenol (12.2 g.) and ethylene oxide (5.5 g.) in ethanol (40 ml.) containing sodium ethoxide (from 0.23 g. of sodium) was heated in a sealed tube at 80° for 4 hr. The solvent was removed and the residual oil was washed in ethereal solution with 2 N-NaOH and then with water, dried, and recovered. Fractionation afforded 2-(3:4-xylyloxy)ethanol as a colourless oil (14.8 g.), b.p. 130 to 134°/1.5 mm., n_D^{25} 1.5331. (ii) The preceding product (8.3 g.) was treated in dry chloroform (50 ml.), containing pyridine (4.0 g.), with thionyl chloride (6.5 g.), and the reaction mixture was boiled under reflux for 2 hr. The solvent was removed and the product was recovered, washed, and dried in ethereal solution. Fractionation afforded 2-(3:4-*xylyloxy*)*ethyl chloride* as a colourless oil (7.25 g.), b.p. 90 to 92°/0.14 mm., n_D^{23} 1.5329 (Found: C, 64.9; H, 7.1. $C_{10}H_{13}OCl$ requires C, 65.0; H, 7.1%).

N-2-(3:4-*Xylyloxy*)*ethylpiperidine Hydrochloride*.—A mixture of 2-(3:4-xylyloxy)ethyl chloride (0.92 g.) and piperidine (0.85 g.) was heated at 140° (bath temp.) for 4 hr. and then partitioned between ether and water. Basic material was extracted by washing the ethereal solution with dilute mineral acid and recovered by basification and further extraction with ether. The crude oily base was converted to the hydrochloride in acetone in the manner outlined above, and recrystallization from methanol/ethyl acetate gave *N*-2-(3:4-xylyloxy)ethylpiperidine hydrochloride as plates (1.0 g.), m.p. 182 to 184° (Table IV, 65).

1-*Bromo*-3-(3:4-*xylyloxy*)*propane*. — A solution of 3:4-xylenol (9.2 g.) in ethanol (38 ml.) containing sodium ethoxide (from 1.73 g. of sodium) was boiled under reflux with 1:3-dibromopropane (45.5 g.) for 4 hr. The solvent was then removed and the product was recovered and washed in chloroform. Fractionation then gave 1-*bromo*-3-(3:4-*xylyloxy*)*propane* as a colourless oil (9.9 g.), b.p. 94°/0.17 mm., n_D^{19} 1.5425 (Found: C, 54.5; H, 6.3. $C_{11}H_{15}OBr$ requires C, 54.3; H, 6.2%) (see Petrow, Stephenson, and Thomas, 1956).

N-3-(3:4-*Xylyloxy*)*propylpiperidine Hydrochloride*. —A mixture of 1-bromo-3-(3:4-xylyloxy)propane (2.43 g.) and piperidine (1.7 g.) in toluene (15 ml.) was boiled under reflux for 2 hr. After cooling, the precipitated piperidine hydrochloride was collected and washed with ether, and the toluene/ether solution was evaporated. The residual oil was treated with dry hydrogen chloride in ether, and recrystallization of the resulting solid from ethanol/ether gave *N*-3-(3:4-xylyloxy)propylpiperidine hydrochloride as colourless plates (1.3 g.), m.p. 170 to 172° (Table IV, 66).

Glycide 3:4-*Xylyl Ether*.—Epichlorhydrin (13.9 g.) and 3:4-xylenol (12.3 g.) were condensed together using the general method of Petrow and Stephenson (1953) and fractionation of the product gave *glycide* 3:4-*xylyl ether* as a colourless oil (9.54 g.), b.p. 122 to 127°/1.3 mm., n_D^{20} 1.5284 (Found: C, 74.2; H, 7.9. $C_{11}H_{14}O_2$ requires C, 74.1; H, 7.9%).

1-*Piperidino*-3-(3:4-*xylyloxy*)*propan*-2-*ol Hydrochloride.*—The preceding glycide ether (1.2 g.) was heated with piperidine (0.62 g., 20% excess) under reflux for 5 hr. Fractionation of the product gave the desired base in practically quantitative yield (1.65 g.) and crystallization from aqueous alcohol gave colourless plates, m.p. 75 to 77°. The hydrochloride, prepared in acetone solution, crystallized from methanol/ethyl acetate in colourless needles, m.p. 171 to 173° (Table IV, 67).

3:4-*Xylylthioacetopiperidide.*—A mixture of 3:4-dimethylacetophenone (3.7 g.), sulphur (1.2 g.), and piperidine (3.2 g.) was heated under reflux in an oil bath for 15 hr. The resulting syrup was extracted with ether and the extract was washed with alkali, acid, and water, and evaporated. The residue was freed from volatile material by steam distillation and again recovered in ether. The residual gum (4.77 g.) solidified on standing and crystallization from *n*-propanol afforded 3:4-*xylylthioacetopiperidide* as colourless rods (2.5 g.), m.p. 83 to 85° (Found: C, 72.8; H, 8.6; N, 5.5. $C_{15}H_{21}NS$ requires C, 72.8; H, 8.6; N, 5.7%).

N-2-(3:4-*Xylyl*)*ethylpiperidine Hydrochloride.*—A mixture of 3:4-xylylthioacetopiperidide (1 g.), Raney nickel (about 3 g.) and ethanol (20 ml.) was boiled under reflux on the water-bath for 5 hr. After removal of the catalyst and solvent, the residual gum was partitioned between ether and dilute HCl. Basic material, recovered in the usual way, was converted to the hydrochloride, and recrystallization from *n*-propanol afforded *N*-2-(3:4-xylyl)ethylpiperidine hydrochloride as colourless plates (0.48 g.), m.p. 261 to 263° (Table IV, 68).

4-*Cyano*-o-*xylene.* — The diazonium salt was prepared below 5° from 3:4-xylidine (24.2 g.), conc. HCl (40 ml.) and sodium nitrite (13.8 g.) in aqueous solution (total vol. about 180 ml.), and added to a hot solution prepared from nickel chloride (47.4 g.), sodium cyanide (49 g.) and water (330 ml.). The product was isolated by steam distillation and ether extraction; the nitrile (19.5 g.) distilled at 116 to 118°/20 mm.

3:4-*Dimethylbenzaldehyde.* — 4-Cyano-*o*-xylene (26.2 g.) in ether (100 ml.) and chloroform (20 ml.) was added to a mixture of anhydrous stannous chloride (56.9 g.) and ether (250 ml.), which had been saturated with dry hydrogen chloride. Next day the aldimine stannichloride was collected and the filtrate was again saturated with dry hydrogen chloride and kept for several days, giving a further crop of solid. The complex was decomposed with hot water and the product was steam distilled and extracted with ether. Fractionation gave 3:4-dimethylbenzaldehyde as an oil (15.8 g.), b.p. 68°/0.8 mm.

β-3:4-*Xylylpropionic Acid.*—(i) A mixture of 3:4-dimethylbenzaldehyde (8.0 g.), malonic acid (6.24 g.) and pyridine (1.4 g.) was heated on the water-bath until evolution of carbon dioxide ceased. The product was purified by solution in aqueous sodium carbonate and reprecipitation; crystallization then afforded 3:4-dimethylcinnamic acid (7.6 g.), m.p. 171 to 173°, in agreement with Sugasawa and Sugimoto (1941). (ii) 3:4-Dimethylcinnamic acid (5.7 g.) was esterified with excess ethereal diazomethane (from 7.5 g. nitrosomethylurea) and the resulting methyl ester was hydrogenated in methanol (100 ml.) in the presence of palladized strontium carbonate at room temperature and atmospheric pressure. The residue obtained after removal of catalyst and solvent was treated with 25% aqueous NaOH (10 ml.) and ethanol (1 ml.) on the water-bath for 10 min. Crystallization of the recovered acid from aqueous ethanol gave β-3:4-*xylylpropionic acid* as colourless needles (4.7 g.), m.p. 82 to 84° (Found: C, 74.1; H, 8.0. $C_{11}H_{14}O_2$ requires C, 74.2; H, 7.8%).

N-3-(3:4-*Xylyl*)*propylpiperidine Hydrochloride.* — (i) The preceding acid (4 g.) was treated in dry chloroform (20 ml.) with thionyl chloride (2.5 ml.) and the mixture was boiled under reflux on the water-bath until evolution of hydrogen chloride ceased. Solvent and excess thionyl chloride were exhaustively removed *in vacuo*, and the residue was treated in dry benzene (20 ml.) with piperidine (4.2 g.). The mixture was heated for 2 hr. after the initial reaction subsided; piperidine hydrochloride was then removed and the filtrate was taken to dryness. The residue was washed in ethereal solution and then afforded an oil (5.1 g.), which slowly crystallized; the *piperidide*, sublimed at 100 to 150°/1.5 mm., had m.p. 35 to 37° (Found: C, 78.2; H, 9.4; N, 5.7. $C_{16}H_{23}ON$ requires C, 78.4; H, 9.4; N, 5.7%). (ii) The preceding piperidide (4.4 g.) was reduced in tetrahydrofuran (40 ml.) with lithium aluminium hydride (1.4 g.) in the manner described above. *N*-3-(3:4-Xylyl)propylpiperidine hydrochloride separated from ethanol/ether in colourless plates (3.3 g.), m.p. 183 to 185° (Table IV, 69).

N-4-(3:4-*Xylyl*)*butylpiperidine Hydrochloride.*—(i) Friedel-Crafts reaction between *o*-xylene (10.6 g.) and succinic anhydride (10 g.) in nitrobenzene in the normal way afforded β-3:4-dimethylbenzoylpropionic acid, crystallizing from aqueous acetic acid in prisms (14.8 g.), m.p. 129 to 131° (Found: C, 69.8; H, 6.7. $C_{12}H_{14}O_3$ requires C, 69.9; H, 6.8%). (ii) Clemmensen reduction (of 14.4 g.) in the normal way gave γ-3:4-xylylbutyric acid as a colourless oil (6.7 g.), b.p. 126°/0.04 mm. (Found: C, 75.0; H, 8.2. $C_{12}H_{16}O_2$ requires C, 75.0; H, 8.3%). Buu-Hoï, Cagniant, Hoan, and Khôi (1950) record a b.p. of 192 to 193° at 12 mm. (iii) The preceding acid (5.7 g.) was converted into the acid chloride and the piperidide in the manner described for the lower homologue, and the piperidide was analogously reduced with lithium aluminium hydride in tetrahydrofuran; *N*-4-(3:4-xylyl)butylpiperidine hydrochloride separated from benzene in colourless plates (2.75 g., 28% overall), m.p. 174 to 176° (Table IV, 70).

Piperidinomethyl 3:4-Xylyl Ketone Hydrochloride.—ω-Chloro-3:4-dimethylacetophenone (1.8 g.) and piperidine (1.7 g.) were heated in benzene (10 ml.) on the water-bath for 5 hr. Basic material was isolated by appropriate partition between aqueous and ethereal phases, and the product was converted to the hydrochloride in the usual way. Recrystallization from ethanol/ether gave piperidinomethyl 3:4-xylyl ketone hydrochloride as needles (0.82 g.), m.p. 177 to 179° (Table IV, 71).

2-Piperidinoethyl 3′:4′-Xylyl Ketone Hydrochloride. — A mixture of 3:4-*dimethylacetophenone* (1.5 g.), piperidine hydrochloride (1.22 g.), paraformaldehyde (4.5 g.), and ethanol (3 ml., containing 1 drop of conc. HCl) was heated under reflux for 1 hr. and for a further 2 hr. after the addition of more paraformaldehyde (0.3 g.). Precipitation of the product was accelerated with acetone (25 ml.), and recrystallization from methanol/ethyl acetate afforded colourless plates (1.1 g.) of the required hydrochloride, m.p. 182 to 184° (Table IV, 72).

N^1N^1-cyclo*Pentamethylene*-N^5-3:4-*xylyldiguanide.*—A mixture of 3:4-xylyldicyandiamide (Curd and Rose, 1946) (4.7 g.), piperidine (1.6 g.), hydrated copper sulphate (1.25 g.), 2-ethoxyethanol (4 ml.) and water (4.5 ml.) was heated under reflux with stirring for 2 hr., when a purple-brown complex separated. After dilution with water (60 ml.) the aqueous phase was decanted, and the residue was treated with N-HCl, when an insoluble residue (1.7 g.) of unchanged xylyldicyandiamide was rejected. The solution was treated with a concentrated solution of sodium sulphide monohydrate (3.5 g.) and charcoal, and filtered. The diguanide, precipitated on addition of excess aqueous NaOH, separated from aqueous ethanol in colourless rods (2.4 g.), m.p. 147 to 150°; the hydrochloride crystallized from methanol/ethyl acetate in colourless rosettes, m.p. 233 to 235° (Table IV, 74).

N^1N^1-cyclo*Tetramethylene*-N^5-3:4-*xylyldiguanide Hydrochloride.*—A mixture of 3:4-xylyldicyandiamide (1.9 g.), pyrrolidine (2 ml.), hydrated copper sulphate (1.25 g.), ethanol (10 ml.) and water (6 ml.) was heated under reflux with stirring for 90 min. The free diguanide, isolated as in the preceding example, was a gum and was converted in ether into the hydrochloride, which separated from methanol/ethyl acetate in colourless needles (2.35 g.), m.p. 243 to 244° (Table IV, 76).

3:4-*Dimethylbenzamidine Hydrochloride.*—A mixture of 4-cyano-*o*-xylene (12.6 g.), chloroform (25 ml.) and ethanol (10 ml.) was saturated with hydrogen chloride at 0° and kept at 2° for 3 days. Solvent and excess hydrogen chloride were removed at room temperature under reduced pressure and the residue was kept at 37° for 5 days with saturated (0°) ethanolic ammonia. Evaporation of the solvent and excess ammonia and recrystallization of the residue from methanol/ethyl acetate gave the amidine hydrochloride as colourless plates (13.6 g.), m.p. 195 to 196° (Table IV, 82).

4:5-*Dimethyl*-2-(3-*piperidinopropylamino*)*thiazole Dihydrochloride.*—A mixture of 3-piperidinopropylamine (see Whitmore, Mosher, Adams, Taylor, Chapin, Weisel, and Yanko, 1944) dihydrochloride (2.1 g.), 3-thiocyanatobutan-2-one (Gregory and Mathes, 1952) (1.3 g.) and water (2.5 ml.) was heated under reflux for 6 hr. Unchanged 3-thiocyanatobutan-2-one was removed by extracting the cooled mixture with ether. The aqueous phase was basified and extracted with ether, affording an oily base. The dihydrochloride, prepared in acetone, separated from ethanol/ether in needles (1.73 g.), m.p. 234 to 236° (Table IV, 85).

Biological Methods

Inhibition of viral synthesis was tested by observing the effect of a substance on the growth of influenza virus in small pieces of chick chorio-allantoic membrane *in vitro*. The method used was based on a modification (Tamm, Folkers, and Horsfall, 1953) of the technique of Fulton and Armitage (1951). Ten- or eleven-day chick embryos were de-embryonated, and the chorio-allantoic membranes cut into small pieces which were then stripped from the underlying shell membrane. The average wet weight of a piece of membrane was about 20 mg. The membrane pieces from a group of eggs were washed and randomized and then placed singly in 6×⅝ in. test-tubes along with 1 ml. of Earle's buffered salt solution (see Parker, 1950). In the buffer were incorporated (*a*) the substance under test, (*b*) 100 units of penicillin, (*c*) the influenza virus, strain MEL (1935) at a dilution of 10^{-3} of a stock seed kept in capillary tubes at −70°; this corresponded to 10^6 to 10^7 times the dose which infected 50% of eggs (ID50). In each test control pieces of membrane without the test substance were included and six tubes were used for each experimental group.

The tubes were stoppered and placed in a roller drum at 37° (8 rev./hr.). After 48 hr. incubation, the fluids were titrated for their haemagglutinin content and the geometric mean titres of each group were calculated. It has been found that a yield in a group of six tubes of 25% or less than that of the controls is highly significant ($P<0.01$) (Isaacs and Lindenmann, 1957). The control and experimental titres were calculated on a $\log_2$ scale, and in this report the observed degrees of inhibitory activity are expressed on a scale in which the symbols have the significance shown in Table I.

In a few control experiments it was shown that the titres of haemagglutinin in the fluids paralleled the infective titres of those fluids, both in the presence and absence of test drugs.

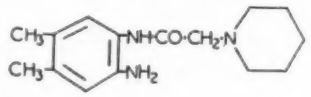
Any toxicity of drugs towards the tissues was shown by characteristic macroscopic changes in the appearance of the membranes (see Tamm, 1956). In control cultures and in the great majority of the drug experiments the membrane pieces appeared healthy at the end of the incubation period. In the presence of some drugs, however, the tissues became white and necrotic-looking, and in a few cases the suspending

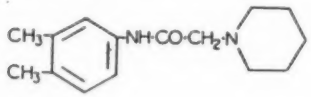
medium became stained a yellowish colour; this was taken as evidence of a severely toxic effect of the drug on the tissue.

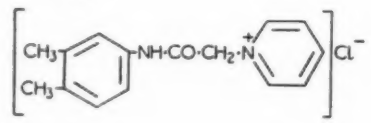
Results

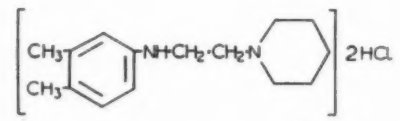
The results of tests for inhibition of virus growth under the conditions described above are recorded in Tables II to IV. It should be pointed out that it is a recognized weakness of the test that substances capable of injuring the tissue would be expected to prevent the tissue from supporting viral growth; such compounds therefore appear to be highly active although the observed inhibitory activity is exaggerated. In the absence of gross toxicity to the tissue, as was observed with the great majority of the compounds tested, the results showed clearly defined differential inhibition of virus growth. Out of 85 compounds tested, nine were obviously toxic to the tissues, three were slightly toxic, and a further three may have been slightly toxic. Four compounds could not be tested satisfactorily, as the free bases were precipitated in the buffered salt solution. Of the remaining 66 compounds, 29 showed highly significant inhibitory activity towards the virus (++, or better).

The first compound examined in the present work, 6-amino-α-piperidinoaceto-3:4-xylidide (IV) dihydrochloride (Table II, 14), completely inhibited the growth of the virus at a concentration of 1 mg./ml. and also at 0.5 mg./ml., but showed no significant inhibitory activity at 0.1 mg./ml., the concentration that was later used routinely.

(IV) 

(V) 

(VI) 

(VII) 

It was immediately noted, however, that α-piperidinoaceto-3:4-xylidide (V) hydrochloride (Table II, 10), the second compound examined, from which the 6-amino group of the compound (IV) is omitted, was considerably more active, and subsequent study was therefore directed principally towards derivatives of the much more accessible aromatic monoamines, rather than towards those of the *o*-diamines. A lengthy series of α-piperidinoacylarylamides was prepared and examined for inhibitory activity towards influenza virus in tissue culture, and these, with the biological results, are recorded in Table II. From this table a number of noteworthy features connecting structure and biological activity are readily apparent.

Of the first 25 compounds, to which the α-piperidinoacetamido group was common, the effects of variation in the aryl group are obvious. The 3:4-xylidide (V) (10) and the *m*-toluidide (4) were of comparably high activity, but omission of the 3-methyl group in compound (V) giving the *p*-toluidide (5) resulted in a fall in activity, and omission of both methyl groups in compound (V) giving the anilide (1) reduced activity still further. No activity under the conditions of the test was detected in the *o*-toluidide (3) or in the 2:6-xylidide (9), suggesting that an *o*-methyl group has an unfavourable effect upon inhibitory activity: again, the 2:5-xylidide (8) was less effective than the *m*-toluidide (4). The remaining three xylidides (6, 7, 11) were of doubtful activity. The relatively low activity of the *p*-chloro derivative (18) was markedly increased by replacing the chlorine atom by bromine (21) and by iodine (22), and replacement of both the methyl groups in the substance (V) (10) by chlorine atoms gave a compound (19) that was definitely toxic to the tissues. In eleven of the remaining compounds in Table II, the 3:4-xylidide moiety was kept constant and the side-chain was varied. It was found that the piperidino group in compound (V) could be replaced by the pyrrolidin-1-yl group (26) and the piperazin-1-yl group (28) with slightly enhanced activity, but, surprisingly, the morpholino compound (27) showed no inhibitory activity under the conditions of the test. The α-piperidinoacetyl group in compound (V) could also be replaced by the α-piperidinopropionyl group (29) and the α-pyrrolidin-1-ylacetyl group (in 26) by the α-pyrrolidin-1-ylpropionyl group (30) with unchanged high activity. The simple α-amino- (32) and α-dimethylamino-aceto-3:4-xylidide (33) were not noticeably active, but the α-dipropylamino- (35), α-diethylamino- (34), and α-diallylamino-aceto-3:4-xylidide (37) were increasingly active in that order.

The purpose of the strongly basic group in the side-chains of the compounds listed in Table II was to ensure solubility in water through the formation of soluble neutral salts. The same object

was achieved by the formation of analogous pyridinium salts which are recorded in Table III. Comparison of the relevant compounds (38 to 49, 51 to 55) with the corresponding compounds having the same aryl group in Table II shows that the same trends that were discernible in the biological activities of the piperidinoacetamido series are again discernible among the pyridinium chlorides. Of the three toluidides (38 to 40) and six xylidides (41 to 46) only the 3:4-xylidide (VI) (45) was found to be outstandingly active. An increase in inhibitory activity was again seen in passing from the *p*-chloro (51) to the *p*-bromo (54) and *p*-iodo (55) derivatives, and the 3:4-dichloro compound (52) was, as in the earlier series, slightly toxic to the tissues. Replacement of the pyridinium group in the compound (VI) by the γ-picolinium (4-methylpyridinium) group resulted in reduced activity (56), and the use of nicotinamide in place of pyridine in the quaternization reaction gave an inactive compound (57). The remaining quaternary ammonium salts (58 to 61) in Table III, obtained by quaternization of bases described in Table II with alkyl halides, were inactive or only feebly active, and it may be noted that the high inhibitory activities of the compound (V) (10) and of the diallylamino compound (37) were abolished (58) or greatly reduced (60) by this procedure.

Attention was then directed towards a study of the permissible variation of the group linking the aryl (3:4-xylyl) and basic (piperidino) groups in the compound (V), in addition to the substitution of the propionyl group for the acetyl group already noted, and the substances examined (62 to 74), together with a number of miscellaneous compounds tested, are recorded in Table IV. In those compounds in which the terminal groups were 3:4-xylyl and piperidino, high activity (and frequently also some toxicity to the tissues) was shown by all compounds in which these two groups were linked through a chain of three or four atoms: this was seemingly irrespective of the nature of the chain. One compound, (N-α-piperidinoacetylglycyl)-3:4-xylidide (73) hydrochloride, with a longer intermediate chain was doubtfully active, and one with a shorter chain (71) was only slightly active. Replacement of the piperidino by the pyrrolidin-1-yl group again gave highly active analogues (75 and 76). The simple N^1-aryldiguanides formed an interesting series, as the 3:4-xylyl representative (80) was fully active but slightly toxic, whereas the *m*-tolyl (77), *p*-tolyl (78), and 2:5-xylyl (79) derivatives were feebly or doubtfully active. 3:4-Xylylguanidine nitrate (81) and 3:4-dimethylbenzamidine (82) were both ostensibly highly active but distinctly toxic to the tissues. Isosteric replacement of the 3:4-xylyl group in the highly active *N*-(3-piperidinopropyl)-3:4-xylidine hydrochloride (63) by the 4:5-dimethylthiazolyl group (in 85) resulted in marked loss of inhibitory activity.

With a view to obtaining more insight into the mode of action of the preceding actively inhibitory compounds, *N*-(2-piperidinoethyl)-3:4-xylidine dihydrochloride (VII) (62) was selected for more detailed examination, as it possessed high intrinsic inhibitory activity towards the virus and had no obvious toxicity for the tissues.

Membranes were incubated under the standard conditions of the test described above with standard inocula of virus and compound (VII) (0.1 mg./ml.), and the virus was harvested 48 hr. later; under these conditions inhibition of the production of haemagglutinin was complete. A further set of membranes from the same large group was incubated with drug alone, and 24 hr. later the tissues were washed with fresh suspending fluid and then exposed to standard inocula of virus and incubated for a further 48 hr.; under these conditions the growth of virus was indistinguishable from that in the control tubes, indicating no permanent damage to the tissue arising from exposure to the drug and no retention of inhibitory amounts of drug by the tissue. In a further experiment the membranes were exposed to drug and virus under the standard conditions of the test. After 24 hr. the tissues were washed and re-suspended in the buffered salt solution plus penicillin; under these conditions the yield of virus 48 hr. later was actually greater than that from the control tubes, indicating effective adsorption of virus by the membranes and inhibition of growth as long as the inhibitory drug was present in the suspending fluid; after removal of the drug by washing uninhibited growth of the adsorbed virus took place.

The possibility was considered that compound (VII) might inhibit the liberation of virus from cells, rather than inhibit viral synthesis. If so, an extract prepared from infected tissue grown in the presence of compound (VII) would contain large amounts of virus. This did not, in fact, occur, but it was observed that such extracts contained complement-fixing "soluble" antigen in greater amount than would be expected from the virus yield. For instance, in one experiment the virus yield was depressed ten-fold whereas the yield of complement-fixing antigen was depressed only two-fold. It is possible, therefore, that compound (VII) exerted its effect on cytoplasmic protein synthesis to a greater extent than on synthesis of the nucleoprotein soluble antigen. Such a

differential effect has also been observed during the inhibition of the growth of influenza virus by caprochlorone in the de-embryonated egg (Liu, Malsberger, Carter, DeSanctis, Wiener, and Hampil, 1957), and a similar effect has been described for proflavine on the growth of another myxovirus, fowl plague virus (R. M. Franklin, personal communication).

Experiments were carried out to see whether the virus-inhibitory activity of the compound (VII) could be reversed by riboflavin or by vitamin B_{12}. Neither riboflavin (approx. 0.02 mg./ml.) nor vitamin B_{12} (0.001 mg./ml.), when added at the beginning of incubation, had any effect in reversing the inhibition of growth produced by the compound (VII) (0.1 mg./ml.). In the presence of 4:5-dimethyl-*o*-phenylenediamine (I) (0.001 mg./ml.), however, the virus-inhibitory activity of compound (VII) (0.1 mg./ml.) was reduced from ++++ to +. At a higher concentration of 4:5-dimethyl-*o*-phenylenediamine (0.01 mg./ml.) no antagonism of the inhibitory action of compound (VII) (0.1 mg./ml.) was observed, and at still higher concentration (0.1 mg./ml.) 4:5-dimethyl-*o*-phenylenediamine itself appeared to be markedly inhibitory (+++) towards the growth of the virus.

At the maximum tolerated dose (about 1 mg./egg) the substance (VII) had no detectable inhibitory effect on the growth of influenza virus in the chick allantoic cavity.

Most of the compounds described were examined for *in vitro* inhibitory activity towards *Streptococcus haemolyticus, Staphylococcus aureus, Escherichia coli,* and *Candida albicans,* but no outstanding activity was observed and no trends relating structure and activity were readily discernible. The majority of the compounds described in Tables II and III were examined for activity against *Trypanosoma cruzi* in the mouse, but no activity was noted.

Discussion

The biological results recorded in the present paper show that the compounds tested possessed an inhibitory action, on the reproduction of influenza virus in the chick chorio-allantoic membrane, that was markedly sensitive to changes in the chemical structures of the compounds concerned. Such inhibition of viral multiplication could be due to a number of causes operating at successive stages in virus production. Thus, (i) a direct inactivation of extracellular virus before its adsorption by the tissues, (ii) the inhibition of adsorption of the virus by the tissue, (iii) inhibition of the multiplication of the virus, or (iv) inhibition of the release of freshly synthesized virus would severally produce the gross result of an inhibition of viral growth, and experiments are described above which effectively dispose of (i), (ii), and (iv) as the causes of the observed inhibition. On the other hand a differential toxic effect on the host tissue could vary the capacity to support viral growth of a tissue in the presence of a range of drugs, but the macroscopic appearance of the tissues in the present work is against such an explanation of the finer differences observable. In all but one of the observed instances of toxicity towards the tissue the inhibition of viral growth was complete, and this particular weakness of the biological test has already been emphasized.

Our conclusion therefore is that those compounds described above, which inhibit viral reproduction without exerting any gross toxic action on the host tissue, inhibit production of viral haemagglutinin and hence production of virus particles. The effect is not due to direct inhibition of haemagglutination, to interference with the start of infection, nor to inhibition of the release of virus from cells. The virus particles produced in the presence of drug seem to be fully infective. There was significantly less inhibition of the production of influenza virus soluble antigen (which is believed to be a nucleoprotein) than of haemagglutinin in the infected tissues. The results, in fact, suggest that the drugs act specifically to inhibit the synthesis of virus haemagglutinin.

It would appear furthermore that the present observations disclose the existence of a biochemical reaction that is of greater importance for the reproduction of the virus than for the normal functioning of the host cell. Having regard to the nature of the compounds concerned and the premises which led to their examination, it was obvious that their mode of action might be related to biochemical events concerned with the functioning of riboflavin (or riboflavin-containing co-enzymes) or of vitamin B_{12}. If an inhibitor, such as compound (VII), blocked the synthesis of one or other of these metabolites the addition of the product of the blocked synthesis might be expected to reverse non-competitively the action of the inhibitor, but neither riboflavin nor vitamin B_{12} appeared to reverse the inhibition of viral growth caused by compound (VII). On the other hand, 4:5-dimethyl-*o*-phenylenediamine (I) caused a marked reduction in the inhibition produced by compound (VII) when present at 1/100th of the concentration of the inhibitor, but was itself inhibitory (or perhaps toxic) at

higher concentration. This observation therefore validates the premises upon which the work was undertaken although it is not possible to define at present the precise biochemical mechanism by which the observed inhibition is mediated. The lack of reversal of the inhibitory effect of compound (VII) in the present system by riboflavin or by vitamin B_{12} and its reversal by 4:5-dimethyl-*o*-phenylenediamine parallel the observations of Woolley and Pringle (1952) on a series of metabolite antagonists based on 4:5-dimethyl-*o*-phenylenediamine (I), modelled on quite different lines from those described in the present communication. One may also note that several of the compounds used by Woolley and Pringle (1952) and related substances have been shown (Gale and Folkes, 1957 ; Gale, 1958) to inhibit the incorporation of glycine by disrupted staphylococcal cells.

The authors are indebted to Dr. A. T. Fuller for the *in vitro* antibacterial tests and to Miss A. E. R. Taylor for carrying out tests for trypanocidal activity.

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THE CURATIVE POWER OF 4-(2:6-DIAMINOPYRIMIDIN-4-YLAMINO)PHENYLARSINE OXIDE (COMPOUND 12,065) IN INFECTIONS OF *TRYPANOSOMA RHODESIENSE* IN LABORATORY ANIMALS

BY

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The toxicity to laboratory animals of 4-(2:6-diaminopyrimidin-4-ylamino)phenylarsine oxide (compound 12,065), and its ability to cure laboratory infections of *Trypanosoma rhodesiense* were investigated. The intramuscular LD50 to mice was 22 mg./kg.; rats were more susceptible. Monkeys tolerated two daily doses of 6 mg./kg., but not two daily doses of 10 mg./kg. The intramuscular CD50 to mice was about 6 mg./kg. with two strains of *T. rhodesiense* recently isolated from human patients, and 2 mg./kg. with a strain of *T. rhodesiense* which had been maintained by cyclical transmission through tsetse flies in the laboratory for about 17 months. These results are compared with those reported by Ainley and Davey (1958) for a strain of *T. rhodesiense* which had been maintained in the laboratory by cyclical transmission for about 12 years and then by syringe transmission for about 10 years. The syringe-transmitted strain was at least 20 times more susceptible to the drug than the recently isolated strains. The potencies of compound 12,065 and melarsen oxide/BAL in curing *T. rhodesiense* infections in mice were compared: the CD50 for compound 12,065 was 6.5 mg./kg. and for melarsen oxide/BAL was 3.7 mg./kg. The ratios LD50/CD50 for the drugs were about 4 and 6 respectively.

Ainley and Davey (1958) and Davey (personal communication) have shown that 4-(2:6-diaminopyrimidin-4-ylamino)phenylarsine oxide (compound 12,065) cured infections of *Trypanosoma rhodesiense* in mice and monkeys, even when the central nervous systems of the monkeys were involved.

Ainley and Davey (1958) used a branch of the Tinde strain of *T. rhodesiense* from Kahama, Tanganyika, which they had maintained by syringe passage in mice for about 10 years, after 12 years of cyclical transmission at Tinde, Tanganyika. Prolonged syringe passage has been shown to alter the susceptibility of trypanosome strains to arsenical drugs (Murgatroyd and Yorke, 1937). Preliminary tests of the compound were therefore made in laboratory animals infected with strains of *T. rhodesiense* which had been maintained by cyclical transmission through tsetse flies and with strains recently isolated from sleeping sickness patients.

MATERIALS AND METHODS

Strains of Trypanosome.—Strain "Lugala II" was isolated from *Glossina pallidipes* in a white rat in September, 1955 (East African Trypanosomiasis Research Organization, 1956). It was maintained, after one initial syringe passage, by cyclical transmission through *G. morsitans* and laboratory guinea-pigs or (occasionally) white rats.

Strain "SS 21" and strain "SS 115" were isolated from human patients by inoculation of blood into white rats (Robertson and Baker, 1958). Experimental animals were inoculated directly from the first isolation rat.

The three strains of *T. rhodesiense* originated from the area on or near the north-east shore of Lake Victoria in the Eastern Province of Uganda.

Experimental Animals.—The animals used were albino rats and mice of the closed colonies of the East African Trypanosomiasis Research Organization at Tororo, and adult monkeys (*Cercopithecus aethiops johnstoni*) trapped in Nzega District, Western Province, Tanganyika, and kept in captivity at Tororo for at least one year.

Drugs.—Compound 12,065 was supplied by Imperial Chemical Industries Limited, Pharmaceuticals Division, as the solid isethionate dihydrate. All doses are given in mg. of the base, one unit of weight of which is equivalent to 1.56 units of the isethionate dihydrate.

Melarsen oxide/BAL (May and Baker), or 4-(3-hydroxymethyl-1-arsa-2:5-dithia*cyclo*pentyl)

benzene (Friedheim, 1950), was supplied as a 3.6% solution in propylene glycol, equivalent to a 2.5% solution of the base.

Infection, Examination, and Treatment of Experimental Animals.—Mice and rats were infected by the intraperitoneal inoculation of infected blood either diluted with citrated normal saline or heparinized and diluted with isotonic glucose solution. Animals in each experiment received equal volumes of diluted blood from the same donor, so that the number of trypanosomes inoculated was approximately equal within each experiment.

Fresh preparations of blood collected from the tails of infected animals were examined daily (except on Sundays). Twenty fields of these preparations were examined under the 4 mm. objective of the microscope and, if no trypanosomes were seen, the animals were classed as negative.

Both drugs were given by intramuscular injection. Infected mice were treated when about five or more trypanosomes/microscope field were seen in the fresh blood preparations, six to eight days after inoculation. Compound 12,065 was dissolved in sterile distilled water to give solutions of from 0.017% to 0.8%. In Expt. No. 6 the concentrations of the drug solutions were adjusted so that each mouse received a volume of 0.05 ml., the melarsen oxide/BAL solution being diluted with propylene glycol. Monkeys and rats were treated on the basis of their individual body weights; mice were treated on the basis of the average body weight of the groups. The criterion of cure adopted was that mice, after the initial clearance of trypanosomes from their blood brought about by the drug, should not relapse within 42 days of treatment.

Treatment of Results.—The lethal and curative doses for 50% of the animals (LD50 and CD50) were calculated by the method of Thompson (1947). In determining the CD50, the mice which died without parasitaemia within 42 days of their treatment were discarded because it was not known whether their deaths were due to sub-patent trypanosomiasis or extraneous causes.

RESULTS

Toxicity Tests.—Seven groups of ten uninfected mice, average weight about 20 g., received single doses of from 2.6 to 25.6 mg./kg. of compound 12,065. The LD50 was about 22 mg./kg. The intramuscular LD50 for mice calculated from the results of Davey (personal communication) was about 39 mg./kg.

Two adult male monkeys, weighing 5 kg., survived total doses of 4 and 12 mg./kg. respectively of compound 12,065 (given in two equal daily doses) with no apparent ill effects. One adult male monkey, weighing 4.5 kg., died 10 days after receiving 20 mg./kg. in two equal daily doses. It

TABLE I

THERAPEUTIC ACTIVITY OF COMPOUND 12,065 AND MELARSEN OXIDE/BAL IN *TRYPANOSOMA RHODESIENSE* INFECTIONS IN WHITE MICE

* Denotes that one of these mice showed trypanosomes in its blood on the 65th day after treatment but was still alive without further parasitaemia on the 100th day.
† Indicates that the mice received 0·05 ml. of normal saline.
‡ Denotes that the mice were given 0·05 ml. of propylene glycol.
§ Indicates that some of these mice (2 receiving 4 mg./kg. and 1 receiving 8 mg./kg.) relapsed between 74 and 92 days after their treament and died with parasitaemia; they are also included in the column headed "Died with Parasitaemia."

Expt. No.	Strain of *T. rhodesiense*	Drug	Dose (mg./kg. of Base)	Number of Mice				CD50 (and Range; $P=0{\cdot}95$) (mg./kg.)
				In Group	Died with Parasitaemia	Died without Parasitaemia Less than 42 Days After Treatment	Survived at Least 42 Days After Treatment Without Parasitaemia (Presumed Cured)	
4	Lugala II	Compound 12,065	0	5	5	0	0	2·0 (0·9–4·6)
			1	4	1	3	0	
			2	5	2	1	2*	
			4	4	0	1	3	
			8	4	0	1	3	
5	SS 21	,,	0	10	10	0	0	5·7 (3·8–8·5)
			0·5	8	2	6	0	
			1	7	6	1	0	
			2	10	7	3	0	
			4	10	5	5	0	
			8	10	0	5	5	
6	SS 115	,,	0†	9	9	0	0	6·5 (3·9–10·8)
			2	8	6	2	0	
			4	9	5	1	3	
			8	7	4	0	3	
			12	10	0	5	5	
		Melarsen Oxide/BAL	0‡	10	10	0	0	3·7 (2·1–6·4)
			2	10	6	2	2	
			4	9	3	3	5§	
			8	10	1	3	7§	
			12	9	0	2	7	

had been ill, with diarrhoea and lassitude, for 5 days and, at autopsy, the kidneys were swollen and haemorrhagic.

Tests of Curative Power.—In all experiments, the blood of treated animals was at least temporarily cleared of trypanosomes: all untreated control animals died with heavy parasitaemia within 37 days of inoculation.

An initial test with rats failed because the animals were very susceptible to the drug; all of those which received 4 mg./kg. or more died, apparently of poisoning.

The results of the tests with mice are set out in Table I. In Expt. No. 6, melarsen oxide/BAL, the drug at present in use for the treatment of advanced cases of *T. rhodesiense* infection (Apted, 1957), was compared with compound 12,065.

Discussion

The intramuscular CD50 of compound 12,065 in mice was about 6 mg./kg. with the two recently isolated strains SS 21 and SS 115, and 2 mg./kg. with the strain Lugala II which had been maintained in the laboratory by tsetse fly transmission for about 17 months. Ainley and Davey (1958) showed that, with their strain of *T. rhodesiense* which had been cyclically transmitted for 12 years and then syringe passaged for about 10 years, the CD50 of compound 12,065 lay below 0.25 mg./kg. for subcutaneous administration: it was probably below this value, and certainly below 0.5 mg./kg., when given intravenously (Davey, personal communication). Thus the recently isolated strains were three times as resistant as the strain cyclically transmitted for 17 months and at least 20 times as resistant as the strain which had been transmitted by cyclical and syringe passage for about 21 years.

The difference between these results and those of Ainley and Davey (1958) is unlikely to have been due solely to the different routes of administration of the drug, because the calculated LD50 (Davey, personal communication) differed by a factor of less than two whether given intravenously, subcutaneously or intramuscularly. The greater resistance of the strains used in the present work could be due to the fact that the mice were treated at rather higher levels of infection than were those of Ainley and Davey (1958) or to the fact that the strain used by these workers had become less resistant during its prolonged maintenance in animals other than man by tsetse fly and syringe transmission. The strain cyclically transmitted for 17 months (Lugala II) and the strains newly isolated from man were treated at about the same levels of infection in the mice. The results suggest that the intensity of infection does not fully explain the difference. Whether the loss of resistance of the strain of Ainley and Davey (1958) was due to the prolonged cyclical transmission or the ten years of syringe transmission is not clear from these results, although the latter procedure is likely to have been at least partly responsible. Syringe transmission has been shown to increase the susceptibility of trypanosome strains to arsenical drugs (Murgatroyd and Yorke, 1937).

The comparison of compound 12,065 and melarsen oxide/BAL shows that the drugs have a similar curative power in infections of *T. rhodesiense* in mice. The CD50 of melarsen oxide/BAL was lower than that of compound 12,065, but the difference was not statistically significant.

The LD50 of compound 12,065 to mice was 22 mg./kg., so the ratio LD50/CD50 (a modification of the therapeutic index, Lourie and Yorke, 1939) was about 4. The toxicity of melarsen oxide/BAL was not investigated, but the results given by Davey (personal communication) show the intravenous LD50 to mice to have been about 22 mg./kg. also; the ratio LD50/CD50 for the latter drug was therefore about 6. A more crucial comparison of the two drugs would be their relative ability to cure monkeys whose central nervous systems were infected with a strain of *T. rhodesiense*. Such tests were attempted in collaboration with Dr. D. H. H. Robertson, but failed because the infected monkeys died almost as soon as involvement of the central nervous system was demonstrable, even when suppressive treatment with suramin was used. A strict parallel between the behaviour of the drugs in mice and men cannot be assumed, but compound 12,065 and melarsen oxide/BAL are likely to be of similar potency in treating advanced cases of human *T. rhodesiense* infection. If so, the possibility of giving the former drug intramuscularly might be an advantage in clinical practice. However, the toxicity of compound 12,065 in man would need careful investigation before clinical use; Apted (personal communication) has obtained some evidence that it may be more toxic to man than melarsen oxide/BAL.

I wish to thank Dr. D. G. Davey, of Imperial Chemical Industries Limited, Pharmaceuticals Division, for his interest in the work, the supply of compound 12,065, and permission to quote from his unpublished report; Dr. F. Hawking, of the Medical Research Council, for his advice; and Dr. F. I. C. Apted, Sleeping Sickness Specialist of the Tanganyika Medical Department, for results of the use of compound 12,065 in man.

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Brit. J. Pharmacol. (1958), 13, 440.

A METHOD FOR RECORDING PERISTALSIS IN ISOLATED INTESTINE

BY

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A method is described for studying peristalsis in isolated pieces of guinea-pig ileum. Records are obtained of longitudinal contractions, intraluminal pressure and volume of fluid expelled.

In the method for recording peristaltic activity recently described by Bülbring and Lin (1958), the two ends of the isolated piece of intestine were held rigidly and no record was obtained of longitudinal contractions. In the method described here the intestine is suspended isotonically and any commercially obtainable isolated organ bath can be used.

Fig. 2 shows the experimental arrangement. A loop of guinea-pig ileum is suspended in an isolated organ bath (50 ml.). The oral end is tied to the short end of a **J** tube (internal diameter 4 mm.). The caudal end is tied to an inverted **U** tube which is fitted with a valve made from flat drainage tubing (" Pauls " tubing obtainable from Philip Harris, Ltd., Birmingham, 3). The inverted **U** tube is suspended from a lever which is suitably counterweighted and carries a frontal writing point. The long limb of the **J** tube is used as a water manometer, transferring the pressure changes in the intestinal lumen by air transmission to a float recorder.

The float is made from a thin-walled bacteriological test tube with the mouth turned in (see inset of Fig. 2). This, and the use of a 1 mm. capillary inlet tube, prevents the two surfaces approaching each other, thus the float does not touch the inlet tube.

Tyrode solution from a Marriotte bottle enters the lumen of the intestine through a thin Portex tube (1.7 mm. internal diameter) into which is inserted a fine polyethylene tube (0.5 mm. internal diameter) to make intraluminal injections with a minimum of dead space. The concentric inner tube reduces the lumen of the outer tube to capillary dimensions, thereby increasing the resistance to the flow of solution, so that the rate of inflow, at a given pressure head, is reduced.

The outer Portex tube is pushed over one end of a small glass **T** piece; the inner tube passes right through and emerges at the opposite end. A seal is effected between the fine inner tube and the glass by using a small specially moulded P.V.C. thimble. The thimble is prepared by dipping a hot steel former into a P.V.C. " latex " (I.C.I. Welvic Paste Type PA/2, see I.C.I. Handbook " Welvic Paste "). The diameters of the former have to be 20% less than the diameter of the tubes which the thimble has to fit.

An electromagnetic vibrator (Fig. 1) is fixed to the vertical rod from which the levers are suspended in order to minimize artifacts due to friction between writing levers and the drum. It is important to note the soft iron pole faces, which are cemented to the magnet with Araldite to prevent rapid deterioration of the permanent magnet.

Fig. 3 is a diagram of the volume recorder developed from that described by Streeten and Vaughan Williams (1951). The fluid is collected

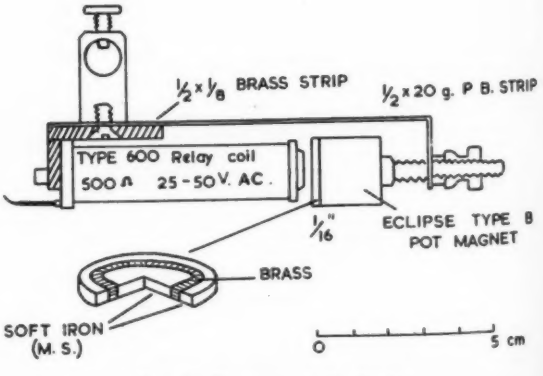

FIG. 1.—Electromagnetic vibrator.

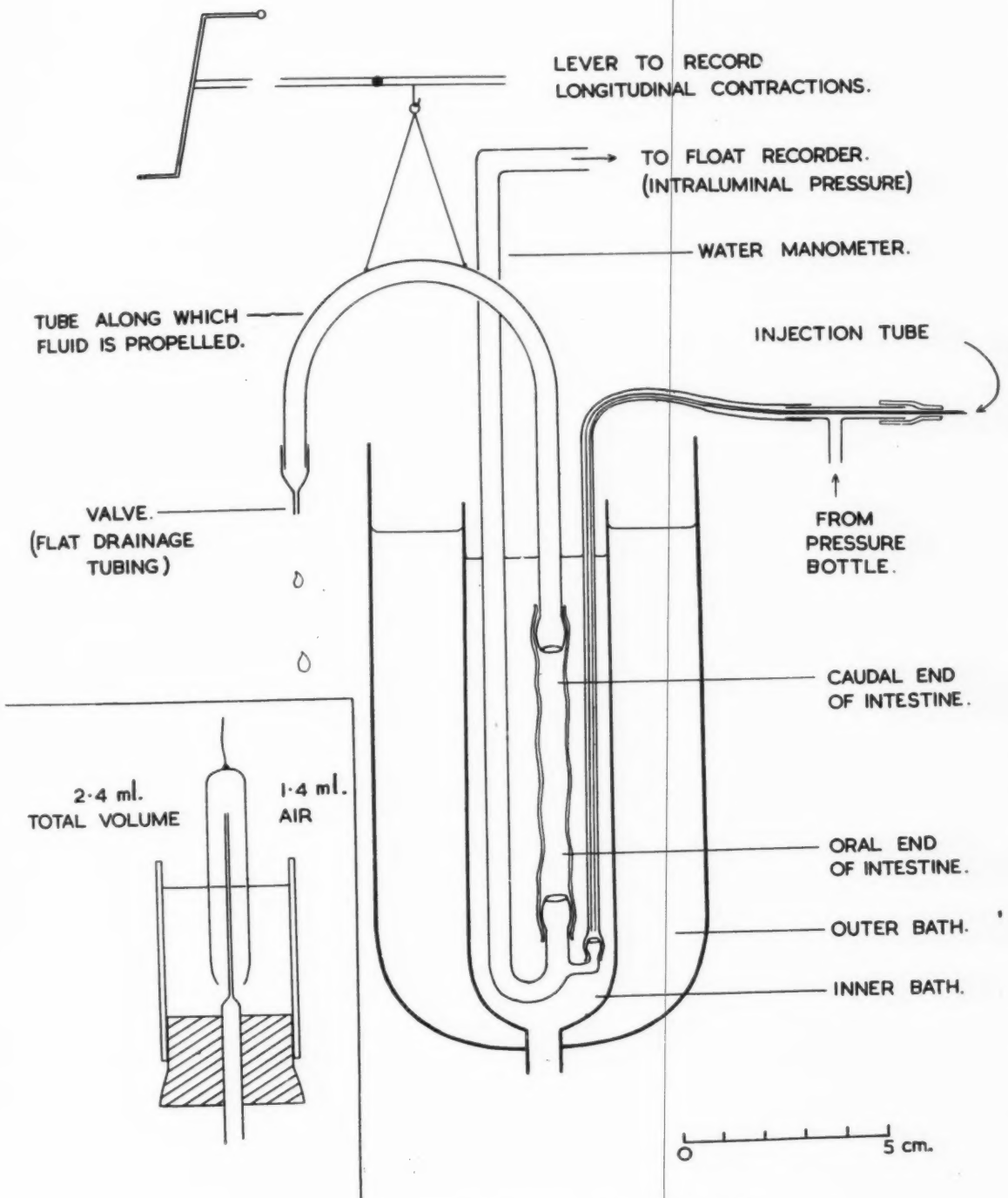

FIG. 2.—General arrangements of apparatus. For description see text.

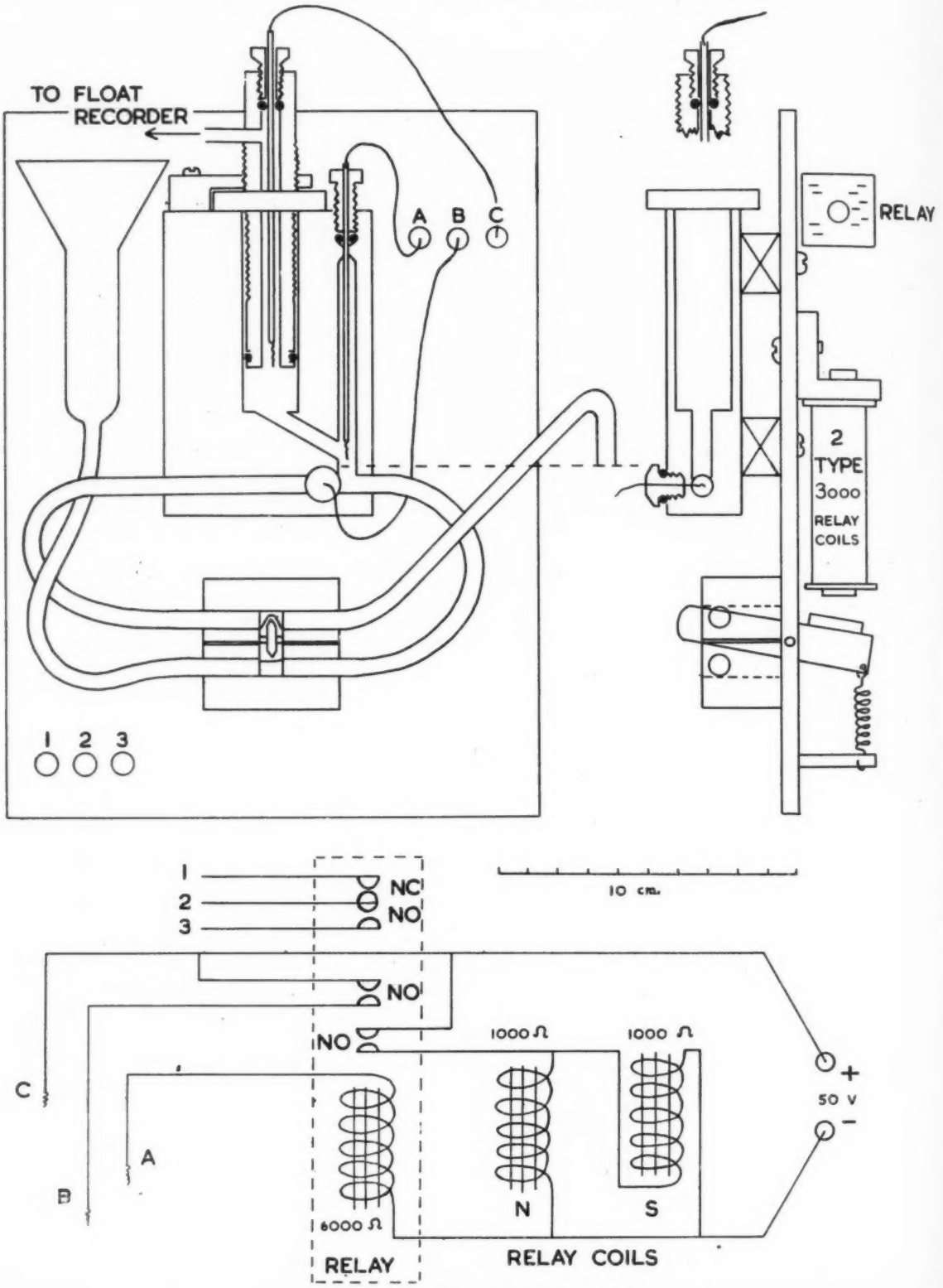

FIG. 3.—Volume recorder. For description see text.

in the funnel and passes into the measuring chamber through an electromagnetic "tap." On entering, the fluid first makes contact with electrode **B**, then with **A**, and finally with **C**. At this stage the "tap" is energized and cuts off the flow, storing any subsequent inflow in the funnel. At the same time it releases the measured volume between **A** and **C**; this escapes through the outlet tube, the mouth of which should be just below the level of **A**. As soon as the fluid falls below **A** the cycle starts again. The electrodes are made of platinum. No disturbance due to polarization is encountered and the apparatus is reliable provided it is kept clean. The volume to be measured (that between **A** and **C**) can be adjusted by moving the piston carrying **C**. This volume can be recorded by air transmission, using a float recorder of somewhat larger volume than that shown in Fig. 2. Alternatively the terminals 1 and 2 or 2 and 3 can be used to operate electrical devices.

A typical tracing obtained with this method is shown in Fig. 4. The upper record (**L**) shows the longitudinal contractions. The middle record (**P**) shows the intraluminal pressure which rises slowly until the peristaltic contraction occurs and empties the lumen. The lower record (**V**) shows the volume of fluid expelled.

This work has been done during the tenure by one of us (A. C.) of a Riker Fellowship for which he wishes to express his thanks to the Riker Laboratories, Ltd. We wish to thank Mr. D. Groves for his assistance.

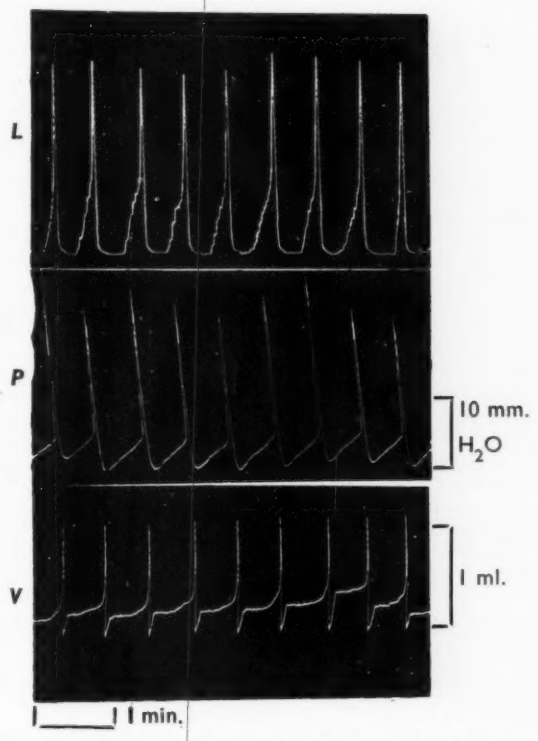

FIG. 4.—Records made from guinea-pig ileum with the apparatus described in the text. *L*, contractions of the longitudinal muscle; *P*, intraluminal pressure; *V*, volume of fluid expelled.

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OBSERVATIONS CONCERNING THE ACTION OF 5-HYDROXYTRYPTAMINE ON THE PERISTALTIC REFLEX

BY

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In isolated guinea-pig intestine 5-hydroxytryptamine increased the longitudinal muscle contractions in response to acetylcholine while the ganglionic action of nicotine was first facilitated and then blocked. Phenyldiguanide, veratrine, veratridine and protoveratrine, like 5-hydroxytryptamine, depressed the response to nicotine, leaving that to acetylcholine unaffected.

The sensory stimulants, like 5-hydroxytryptamine, facilitated the peristaltic reflex when applied to the mucosa, and abolished it when applied to the serosa. Preceding the block, the initial effect of low concentrations of 5-hydroxytryptamine applied to the serosa was a short stimulation of peristalsis.

Concentrations of 5-hydroxytryptamine which had an approximately equal stimulant action (mucosal 1 to 4 $\times$ 10^{-6}, serosal 2 to 8 $\times$ 10^{-8}) were tested when various parts of the reflex arc were blocked. During block by procaine introduced into the lumen, mucosal application of 5-hydroxytryptamine re-established peristalsis, but serosal application of 5-hydroxytryptamine had no effect. During block by hexamethonium or atropine present in the bath, 5-hydroxytryptamine restored peristalsis more effectively by serosal application than by mucosal application. During block by serosal application of 5-hydroxytryptamine, morphine, phenoxybenzamine or dihydroergotamine, mucosal application of 5-hydroxytryptamine restored the peristaltic reflex while serosal application had no effect. During block by 2-bromo-lysergic acid diethylamide or lysergic acid diethylamide acting from the serosal surface, 5-hydroxytryptamine had no effect whether acting on the mucosal or on the serosal surface.

It is concluded that 5-hydroxytryptamine facilitates the peristaltic reflex at two sites: when introduced into the lumen it stimulates mucosal sensory receptors; when acting from the serosal surface it sensitizes the muscle to the transmitter acetylcholine. There is also a transient stimulant action on the ganglia which is soon followed by inhibition; this indicates that 5-hydroxytryptamine applied to the serosa abolishes peristalsis by ganglion block.

Two different actions of 5-hydroxytryptamine on the peristaltic reflex have recently been observed on isolated intestine of the guinea-pig. If 5-hydroxytryptamine was applied to the serosal surface, it inhibited peristalsis (Kosterlitz and Robinson, 1957; Ginzel, 1957); if it was applied to the mucosal surface it facilitated the peristaltic reflex (Bülbring and Lin, 1958; Lembeck, 1958). The stimulant action could be explained by the effect of 5-hydroxytryptamine on sensory receptors in the mucosa. The mechanism of the inhibitory action, however, when 5-hydroxytryptamine was applied to the serosa was not clear.

It is known that a variety of substances stimulate not only chemoreceptors but also tension and stretch receptors in various organs (Dawes and Comroe, 1954; Iggo, 1957; Paintal, 1954, 1957a and b). In the present investigation the action of some sensory stimulants, phenyldiguanide and veratrum alkaloids, has been compared with that of 5-hydroxytryptamine. Furthermore, the action of 5-hydroxytryptamine was tested in the presence of various substances which blocked different parts of the peristaltic reflex arc selectively, in an attempt to obtain more information on the site of action of 5-hydroxytryptamine.

METHODS

In one series of experiments the longitudinal contractions of isolated strips of guinea-pig ileum were recorded in the conventional way. In a second series of experiments the peristaltic reflex was studied, using the method of Bülbring and Lin (1958) and also a modification described by Bülbring, Crema and

Saxby (1958). By this method records were obtained of the longitudinal contractions, the intraluminal pressure, the threshold at which the peristaltic reflex was elicited, and of the fluid expelled from the lumen during peristalsis. Normally, no fluid flowed unless there was peristaltic activity. However, in experiments in which peristalsis was blocked and in which it was desired to test the effect of intraluminal application of various substances, the height of the fluid reservoir in relation to the outflow valve was adjusted in such a way that a slow flow of solution was maintained.

We used pure samples of veratridine and protoveratrine, a crude preparation of veratridine (B.D.H.), and veratrine (mixture of alkaloids) [B.D.H.]. Doses of 5-hydroxytryptamine are expressed in weight of 5-hydroxytryptamine creatinine sulphate (Sandoz, Ltd.).

Results

The Effect of 5-Hydroxytryptamine, Phenyldiguanide and Veratrine on the Longitudinal Contractions of Isolated Guinea-pig Ileum Evoked by Acetylcholine and Nicotine

Feldberg and Lin (1949) used small doses of acetylcholine and nicotine to produce contractions of the isolated intestine in order to test the excitability of the muscle and ganglia respectively. We have used their method to investigate the action of 5-hydroxytryptamine and other substances which are known to be sensory stimulants. Acetylcholine in concentrations between 1 and 2×10^{-8} and nicotine between 2 and 8×10^{-6} produced contractions of about equal size in isolated strips of guinea-pig ileum. We found that 5-hydroxytryptamine sensitized the muscle to the action of acetylcholine but reduced the action of nicotine on the ganglia.

Fig. 1 shows the effect of a short exposure to three different concentrations of 5-hydroxytryptamine on (*a*) the response to acetylcholine, and (*b*) the response to nicotine. Both were increased. It may be noted that in Fig. 1 (*a*) the response to acetylcholine was increasingly augmented with higher concentrations of 5-hydroxytryptamine in spite of the tachyphylaxis to 5-hydroxytryptamine itself, the largest dose of which had no effect. Fig. 1 (*b*) shows that the response to nicotine was more increased by 5-hydroxytryptamine 2×10^{-7} than by 2×10^{-8}, but less increased by 2×10^{-6}. Larger doses of 5-hydroxytryptamine caused only depression.

Fig. 2 (*a*) shows the effect of prolonged exposure to 5-hydroxytryptamine 2×10^{-5}. This caused a large contraction, but the muscle relaxed completely while 5-hydroxytryptamine was still

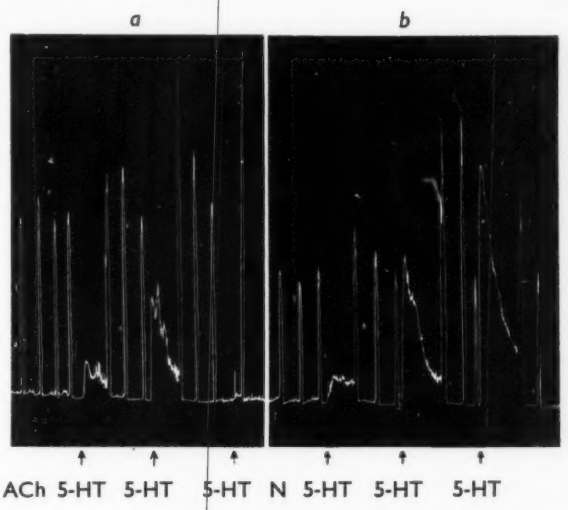

FIG. 1.—Longitudinal contractions of isolated guinea-pig ileum. Effect of 5-hydroxytryptamine (*5-HT*) (1) 2×10^{-8}, (2) 2×10^{-7} and (3) 2×10^{-6} on (*a*) response to acetylcholine (*ACh*) 1×10^{-8}; (*b*) response to nicotine (*N*) 2×10^{-6}.

present. In this condition the acetylcholine response was increased, while the nicotine response was depressed. When 5-hydroxytryptamine was washed out, the acetylcholine response declined and the nicotine response recovered slowly, but complete recovery occurred only after 45 min.

Thus in all concentrations from 2×10^{-8} to 2×10^{-5} 5-hydroxytryptamine increased the action of acetylcholine; it never decreased it. On the other hand, though low concentrations of 5-hydroxytryptamine enhanced the nicotine response, this soon gave way to depression. Higher concentrations of 5-hydroxytryptamine regularly reduced the effect of nicotine.

Phenyldiguanide, tested in the same way as 5-hydroxytryptamine, did not increase the response to acetylcholine or nicotine. In concentrations below 2×10^{-5} it had no effect on the acetylcholine response, but depressed that to nicotine; in doses of 4 and 8×10^{-5} it depressed both, but the response to nicotine was reduced to a greater extent than that to acetylcholine. Moreover, the depression of the acetylcholine response by phenyldiguanide was very transient. This is probably the reason why it was not observed previously by Bülbring and Lin (1958), who only noticed the depression of the nicotine response.

In Fig. 2 (*b*) an experiment is shown in which phenyldiguanide 4×10^{-5} was present for 1 hr. The responses to both acetylcholine and to nicotine were at first reduced by half. But while the nicotine response was progressively further depressed the acetylcholine response soon recovered though phenyl-

diguanide was still present. The nicotine response recovered only 30 min. after washing out the phenyldiguanide.

A few experiments were carried out using veratrine which, in concentrations of 1 and 2×10^{-6}, depressed the response to acetylcholine as well as to nicotine but, like 5-hydroxytryptamine and phenyldiguanide, had a stronger depressant effect on the nicotine contraction.

From the results described so far it appeared that 5-hydroxytryptamine had a selective depressant action on ganglionic excitability but increased the muscular response to acetylcholine. Phenyldiguanide had a similar differential action, depressing the ganglionic response severely but reducing the muscle response only temporarily. Further experiments were therefore carried out in which the peristaltic reflex was used to test the action of sensory stimulants on the nervous mechanism.

The Effect of Phenyldiguanide, Nicotine, and Veratrum Alkaloids on the Peristaltic Reflex

In the method described by Bülbring and Lin (1958) the preparation was suspended isometrically. This method was only used for a few

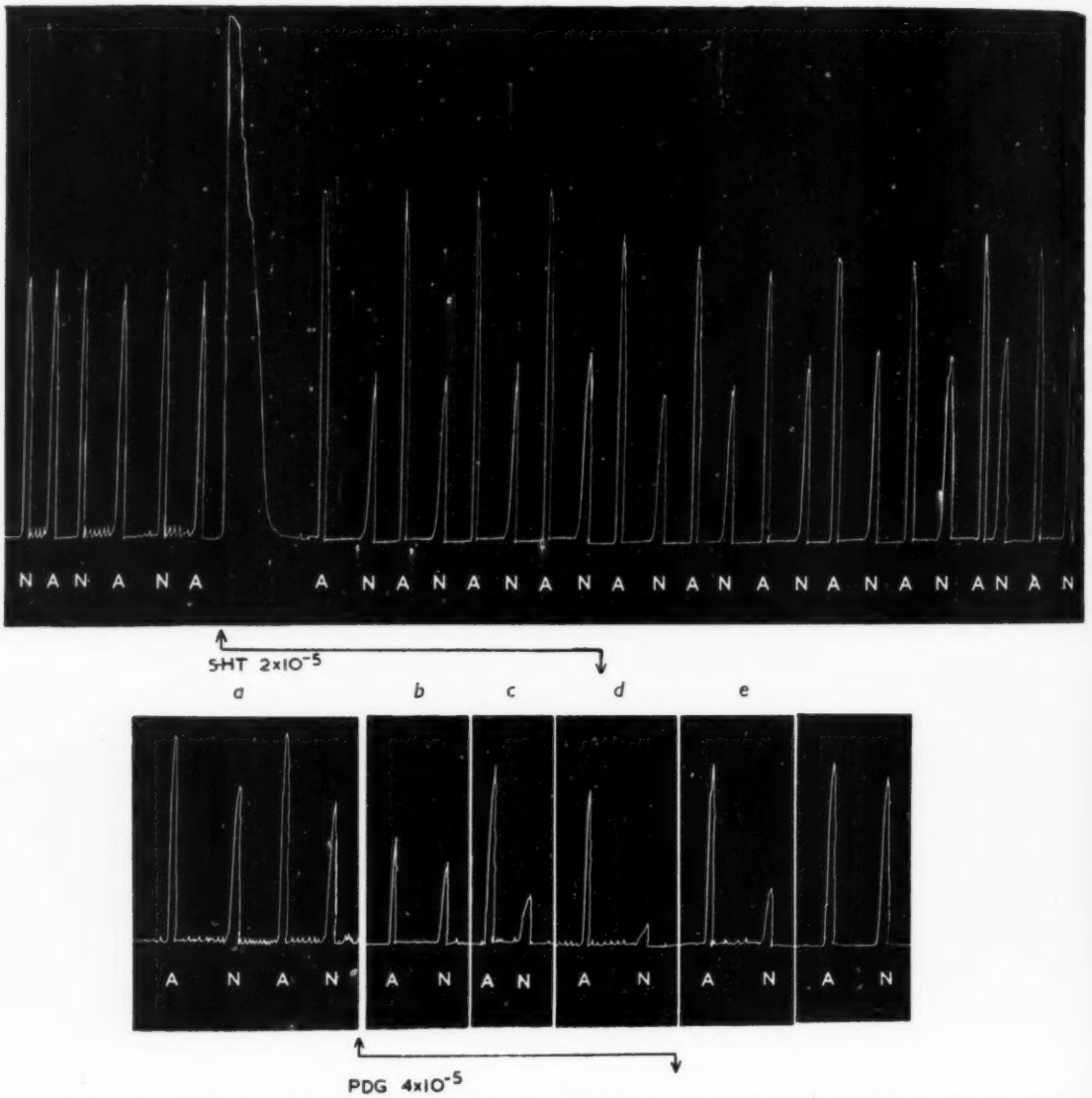

FIG. 2.—Longitudinal contractions of isolated guinea-pig ileum caused by acetylcholine (*A*) $2{\cdot}5 \times 10^{-8}$ and by nicotine (*N*) 4×10^{-6}. Upper tracing: between the arrows 5-hydroxytryptamine (5-HT) 2×10^{-5} was present. Lower tracing: between arrows phenyldiguanide (PDG) 4×10^{-5} present. (*a*) before PDG added; (*b*) 9 min., (*c*) 24 min., (*d*) 51 min. after PDG added., (*e*) 6 min., (*f*) 24 min. after PDG removed.

experiments. It was modified (Bülbring *et al.*, 1958) in order to record longitudinal contractions in isotonic conditions as in the original method described by Trendelenburg (1917). In addition fluid was passed through the lumen so that the intraluminal pressure, the threshold at which the peristaltic reflex occurred, and the fluid transport could also be recorded.

The substances tested all acted like 5-hydroxytryptamine when they were administered into the intestinal lumen. They first caused facilitation of the peristaltic reflex, and this was followed by block. An example is shown in Fig. 3. The record of intraluminal pressure (P) was taken from the oral end of the intestine, showing the slow rise of filling pressure and (at the dot) the steep rise due to the peristaltic contraction. After the injection of 50 μg. protoveratrine the threshold was reduced and thus the frequency of contractions increased. The change of pressure during each contraction became smaller, as there was less back pressure and more fluid was pushed in the caudal direction. This was evident from the lower record (V) in which the vertical strokes, indicating the expulsion of 1 ml. of fluid, occurred at shorter intervals. When the stimulant action gave way to paralysis, the back pressure increased while fluid propulsion diminished and finally stopped. The paralysis which followed the initial stimulation was observed in spite of the fact that in most experiments the substances to be tested were given by injection at a short distance from the point at which the fluid entered the lumen of the intestine (which represented a dead space of 1 ml.). This method allowed the drug to exert its action only for a short time after which it was removed by the constant flow of solution. All the results are summarized in Table I.

Serosal application of the sensory stimulants blocked peristalsis. Phenyldiguanide in doses of 0.2 to 0.4 mg., producing a concentration of 4 to 8×10^{-6} in the bath, depressed the peristaltic reflex. Lower concentrations had no effect, higher concentrations abolished the reflex. Fig. 4 shows the effect of adding to the bath in (*a*) 0.4 mg. phenyldiguanide which reduced the longitudinal muscle contractions and raised the pressure threshold. When 1.0 mg. phenyldiguanide was added to the bath in (*b*) a spasm occurred and peristalsis stopped, except for some irregular waves which occurred at a high threshold.

Veratrine and the pure veratrum alkaloids de-

TABLE I

EFFECTS ON PERISTALTIC REFLEX OF ISOLATED GUINEA-PIG ILEUM

(+ = stimulation, − = depression)

Bath vol. = 50 ml. Intestinal lumen, c. 2 ml., continuously changing during peristalsis at c. 2 ml./min.

Substance	Mucosal Application		Serosal Application	
	Dose Giving Effect (Equivalent to 100 μg. 5-Hydroxytryptamine)	Effect	Dose	Effect
Phenyldiguanide	0·2 mg. injected	+ −	0·2–0·4 mg.	Depression, threshold raised
,,	4×10^{-5} infused	+ −	2·0 mg.	Contracture, reflex abolished
Veratrine complex	150 μg.	+ −	Not tested	
Veratridine crude	100 μg.	+ −	,,	
Veratridine pure	50 μg.	+ −	5–10 μg.	Irregular contractions, reflex impaired
			50 μg.	Peristalsis abolished
Protoveratrine	50 μg.	+ −	5–10 μg.	Irregular contractions, reflex impaired
			50 μg.	Peristalsis abolished
Nicotine	20–30 μg.	+ −	20 μg.	+ −

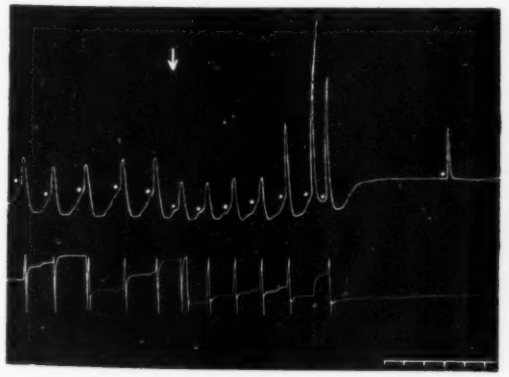

FIG. 3.—Effect of an intraluminal injection (at arrow) of 50 μg. of protoveratrine on peristalsis. Method of Bülbring and Lin (1958). Upper trace: intraluminal pressure (*P*). Threshold is marked by dots. Lower trace: volume (*V*). Each vertical stroke indicates the expulsion of 1 ml. fluid from the isolated gut. Time, 30 sec.

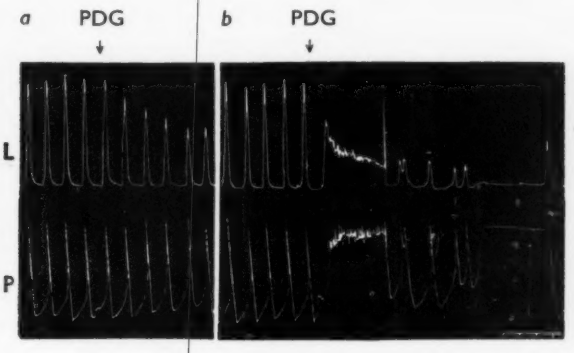

FIG. 4.—Effect of serosal application of phenyldiguanide (PDG) on peristalsis. Method of Bülbring *et al.* (1958). Upper trace: longitudinal contractions (*L*). Lower trace: intraluminal pressure (*P*). At first arrow 0·4 mg. PDG and at second arrow 1 mg. PDG was added to fluid in the 50 ml. bath. Time, 30 sec.

pressed or abolished the peristaltic reflex when they were applied to the serosal surface. They caused irregular, incoordinated muscle contractions, and consequently fluid propulsion was impaired. When veratrine or the pure veratrum alkaloids were washed out, after serosal as well as mucosal application, there followed a prolonged period of increased activity with frequent contractions at very low pressure threshold; yet the volume of fluid expelled was diminished. While this activity persisted, further veratrine doses were ineffective. Furthermore, if veratrine or the pure veratrum alkaloids were given in gradually increasing doses, starting with subthreshold doses, no effect was produced by large doses which otherwise, if applied as the first dose, were effective.

From these experiments it appeared that phenyldiguanide and the veratrum alkaloids, like 5-hydroxytryptamine, caused sensitization followed by desensitization of mucosal sensory receptors when the substance in question was introduced into the lumen of the intestine.

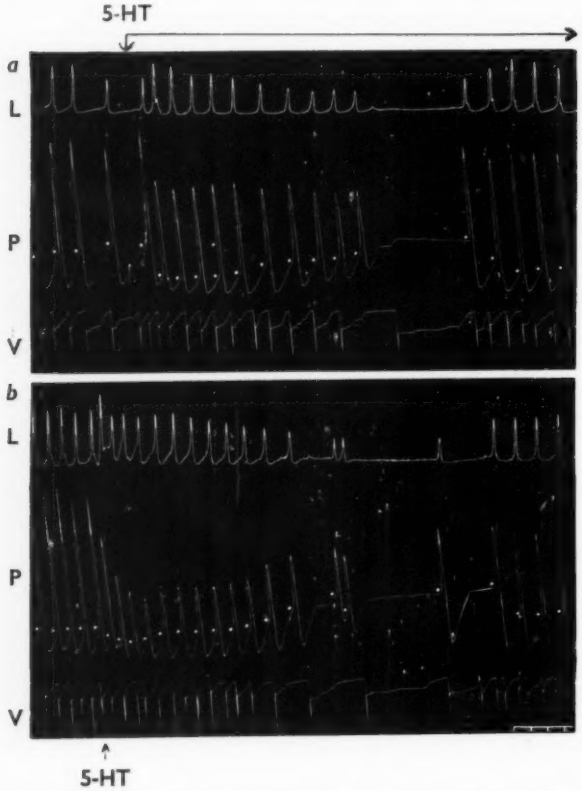

FIG. 5.—Effect of 5-hydroxytryptamine (5-HT) on peristalsis. Method of Bülbring *et al.* (1958). Upper trace: longitudinal contractions (*L*). Middle trace: intraluminal pressure (*P*). Threshold is marked by dots. Lower trace: Volume (*V*). Each vertical stroke indicates expulsion of 1 ml. fluid. (*a*) From arrow, mucosal application of 5-HT: concentration of 4×10^{-6} in solution passing through lumen. (*b*) At arrow, serosal application of 5-HT 2×10^{-7} (10 μg. added to 50 ml. bath). Time, 30 sec.

Serosal application of the sensory stimulants depressed the peristaltic reflex as did 5-hydroxytryptamine; but unlike 5-hydroxytryptamine they produced no initial facilitation.

The Action of 5-Hydroxytryptamine on the Normal Peristaltic Reflex

In the present work, in which the isolated piece of intestine was suspended in isotonic conditions, it was found that 5-hydroxytryptamine, when applied to the serosal surface, could in low concentrations (10^{-8} to 10^{-7}) also stimulate peristalsis. This effect has been described for cooled intestine by Beleslin and Varagić (1958). The stimulation was short-lasting but rapid in onset. The effect of intraluminal application of 5-hydroxytryptamine 4×10^{-6} is shown in Fig. 5*a*, and the response to the addition of 10 μg. 5-hydroxytryptamine to the solution in the bath, producing a concentration of 2×10^{-7}, is seen in Fig. 5*b*. Both caused stimulation followed by paralysis. Acting on the mucosa (*a*), 5-hydroxytryptamine lowered the threshold considerably for a period of 6 min., and after a short pause in activity the threshold remained lower than before. Acting on the serosal surface (*b*), 5-hydroxytryptamine lowered the threshold slightly for about 2 min., and, after a pause, the threshold remained higher than before. It should be noted that as the propulsive efficiency was increased the development of back pressure during each contraction, as measured at the oral end of the intestine, in the middle trace, became less. Fig. 6 shows, for comparison, the effects of two different doses of 5-hydroxytryptamine added to the bath. The contracture of the longitudinal muscle and the diphasic action on the threshold of intraluminal pressure and on the fluid transport are clearly seen.

With 5-hydroxytryptamine, as with veratrine, it was difficult to determine threshold doses because, when gradually increasing doses were administered, desensitization took place and no effect was produced by large doses. Many experiments were carried out giving doses in ascending as well as in descending order. As a result it was found that the concentrations of 5-hydroxytryptamine which had an approximately equal stimulant action on peristalsis were 4×10^{-6} (intraluminal) and 8×10^{-8} (serosal). These concentrations were used for testing the activity of 5-hydroxytryptamine in a series of experiments in which the peristaltic reflex was blocked at different parts of the nervous pathway.

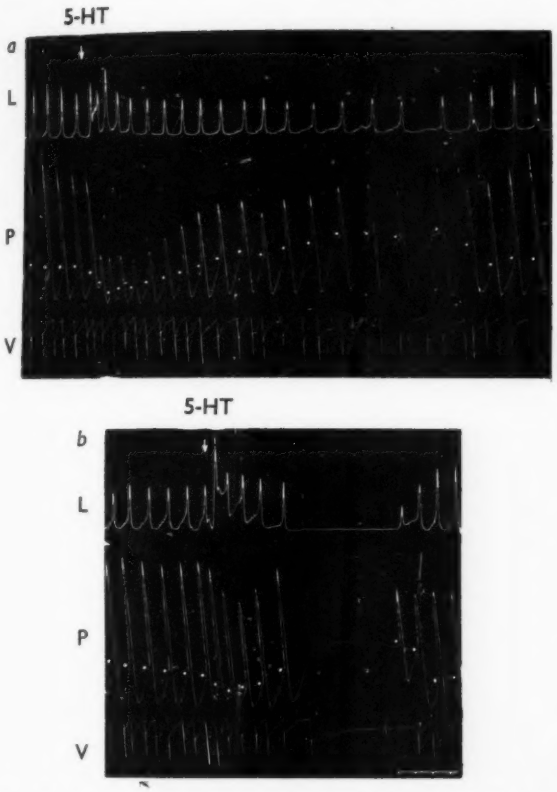

FIG. 6.—Records as in Fig. 5. Effect of serosal application of 5-hydroxytryptamine (5-HT). At arrow, (a) 5 μg., (b) 20 μg. 5-HT added to 50 ml. bath. Time, 30 sec.

The Action of 5-Hydroxytryptamine on the Peristaltic Reflex in the Presence of Procaine in the Intestinal Lumen

Procaine was used to block the mucosal sensory receptors. It was added to the fluid passing through the intestinal lumen in concentrations of 4 to 8×10^{-5}, which produced partial block of peristalsis, raising the threshold and slowing the contractions. Occasionally complete block of the peristaltic reflex occurred. Fig. 7 shows that, in the presence of procaine, 5-hydroxytryptamine applied to the mucosa was able to overcome the partial block, but that serosal application of 5-hydroxytryptamine had no effect. (Phenyldiguanide 1×10^{-4} applied to the mucosa was similarly active but weaker than 5-hydroxytryptamine.) The concentrations of procaine used for these experiments never exceeded 1×10^{-4}. When complete block occurred, mucosal application of 5-hydroxytryptamine and phenyldiguanide still caused peristalsis to reappear though only after a latent period of 3 to 6 min.

The results suggested that, when procaine was present in the lumen, stimulation of the partially or completely depressed sensory receptors by mucosal application of 5-hydroxytryptamine or phenyldiguanide restored the reflex, but that stimulation of muscles and ganglia did not.

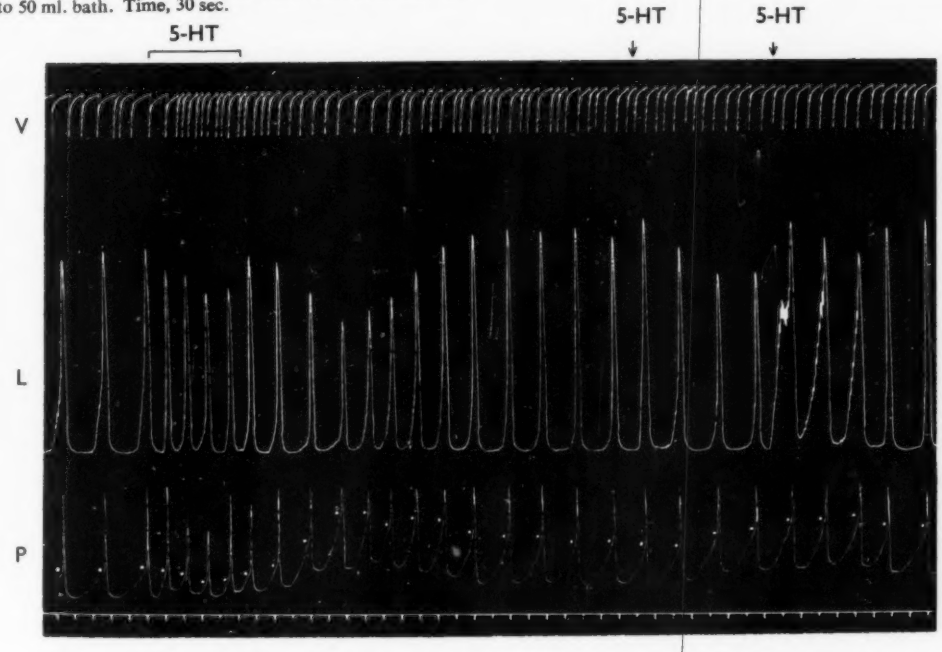

FIG. 7.—Records as in Fig. 5. In presence of procaine 8×10^{-5}, addition of 4×10^{-6} 5-hydroxytryptamine (5-HT) to solution passing through the lumen (indicated by the bracket) lowered threshold and increased volume of fluid expelled. Addition to 50 ml. bath of 4 μg. and 10 μg. of 5-HT (arrows) had no effect on peristalsis. Time, 30 sec.

The Action of 5-Hydroxytryptamine on the Peristaltic Reflex in the Presence of Hexamethonium and Atropine Acting from the Serosal Surface

Hexamethonium was used to block the peristaltic reflex path at the ganglia. A concentration of 3×10^{-4} usually produced partial block and 6×10^{-4} abolished peristalsis. When the block was not complete, 5-hydroxytryptamine, whether applied to the mucosa or to the serosal surface, was about equally active in facilitating the reflex. This is illustrated in Fig. 8. When peristalsis was abolished by hexamethonium, mucosal application restored the reflex only after a latent period of 3 to 5 min. (Fig. 9*a*). Repeated doses of nicotine, reaching a concentration of 4×10^{-7} in the bath, also overcame the blocking action of hexamethonium (Fig. 9*c*). In this record the vigorous pendular activity of the longitudinal muscle should be noted which occurred in the presence of hexamethonium during prolonged distension of the intestine; peristaltic activity, however, occurred only after 5-hydroxytryptamine or nicotine was added.

Atropine was used to block the action of the nervous transmitter acetylcholine on the muscle. Concentrations between 2×10^{-9} to 5×10^{-7} were used to depress peristalsis (Fig. 10); the threshold became higher and the longitudinal contractions became smaller. In these conditions mucosal and serosal application of 5-hydroxytryptamine were about equally effective in lowering the threshold. The addition of methacholine to the bath to produce a concentration of 2×10^{-9} to 1×10^{-8} was followed by a strong contraction and lowered the threshold at which the peristaltic reflex occurred (Fig. 10*b*). When the atropine concentrations were raised and the action of previously effective doses of methacholine was abolished, 5-hydroxytryptamine was still active, both with mucosal and serosal application (Fig. 10*c*). Very high concentrations of atropine, up to 1.2×10^{-5}, were required to abolish the peristaltic reflex. In this condition mucosal application of 5-hydroxytryptamine had no effect, but Fig. 10*d* shows that serosal application of only 4 μg. of 5-hydroxytryptamine was still effective in overcoming the block. The dose of methacholine (Fig. 10*d*), however, had to be increased to 1,000 times that which stimulated peristalsis before atropine was given. It produced a contracture of the longitudinal muscle lasting for $2\frac{1}{2}$ min. before violent contractions of the circular muscle occurred. In general, during complete block of peristalsis by atropine, as with hexamethonium, mucosal application of 5-hydroxytryptamine was less effective than serosal application.

The results obtained in the presence of hexamethonium and atropine suggested that, when the reflex pathway was blocked either at ganglionic or at muscular sites, the stimulation of these sites by serosal application of 5-hydroxytryptamine restored the reflex. When peristalsis was only partially blocked, the stimulation of sensory receptors in the mucosa also restored the reflex; but this was less effective than the action of 5-hydroxytryptamine at ganglionic and muscular sites. Further experiments were therefore carried out to ascertain that the two effects of 5-hydroxytryptamine were in fact due to two distinct actions at two different sites.

The Action of 5-Hydroxytryptamine, Applied to the Mucosa, on the Peristaltic Reflex in the Presence of 5-Hydroxytryptamine Acting from the Serosal Surface

When small doses of 5-hydroxytryptamine were repeatedly added to the bath, the first dose caused, as described above, some stimulation, but subsequent doses caused inhibition, and peristaltic activity ceased. During this block, which was caused by 5-hydroxytryptamine present in the bath (concentration about 5×10^{-7}), it was possible to restore the peristaltic reflex by introducing 5-hydroxytryptamine into the lumen of the intestine.

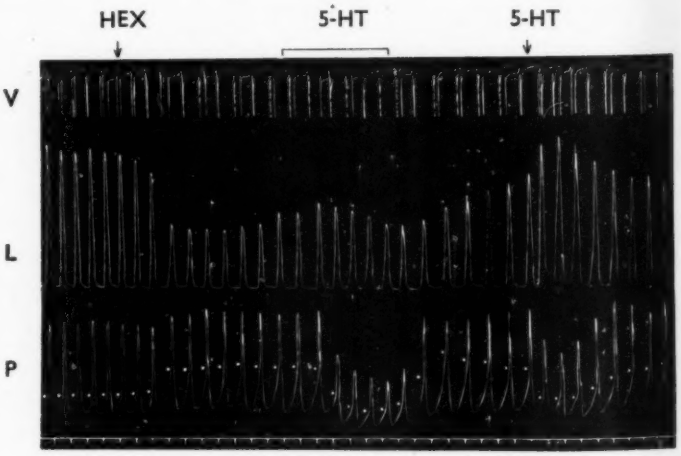

FIG. 8.—Records as in Fig. 5. At first arrow addition of 150 μg. hexamethonium to 50 ml. bath reduced longitudinal contractions (*L*) and raised pressure threshold (*P*). Mucosal application (indicated by the bracket) of 4×10^{-4} of 5-hydroxytryptamine (5-HT) had a stronger effect on threshold and at second arrow serosal application of 8×10^{-8} of 5-HT had a stronger action on longitudinal muscle contractions. Time, 30 sec.

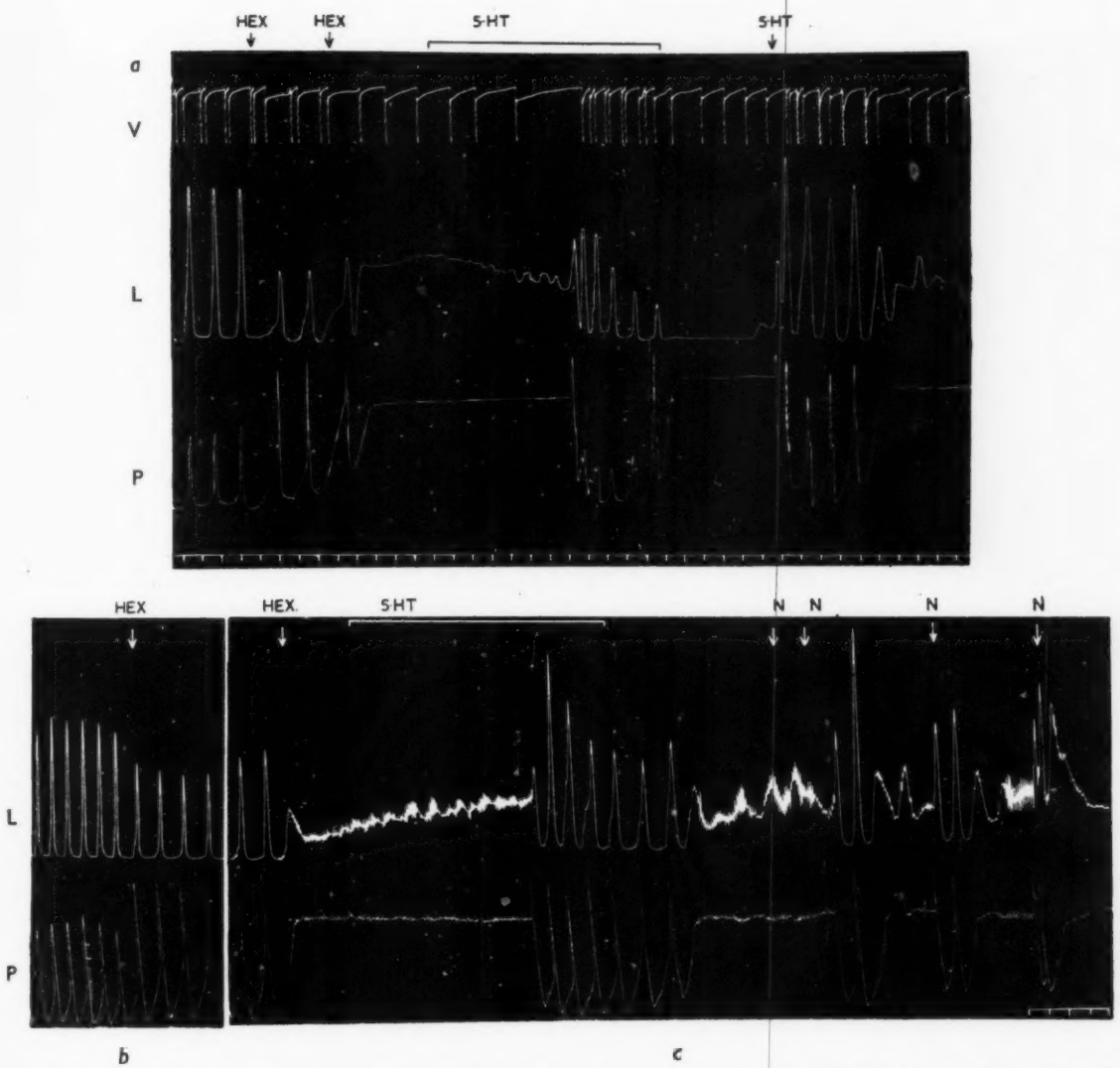

FIG. 9.—Records as in Fig. 5. (*a*) Complete block of peristalsis by two doses of hexamethonium (***HEX***) of 300 μg. each, to 50 ml. bath. Intraluminal 5-hydroxytryptamine (***5-HT***) indicated by the bracket restored reflex after 3 min. latent period, addition to bath (arrow) had immediate effect. Note increased fluid volume expelled. (*b*) Partial block caused by addition of 150 μg. of ***HEX***. (*c*) Complete block caused by further addition of 300 μg. of ***HEX*** to the bath. Reflex restored by mucosal application of ***5-HT*** during the bracket and also by serosal application of nicotine (***N***) at the arrows of 20 μg., 20 μg., 20 μg., and 40 μg. into 50 ml. bath. Time, 30 sec.

Such an experiment is shown in Fig. 11. The first dose of 16 μg. of 5-hydroxytryptamine, applied to the serosa, caused stimulation: subsequent doses of 4 μg. became gradually ineffective and peristalsis stopped. However, mucosal application caused peristalsis to reappear (Fig. 11*a*) after a latent period of several minutes. Peristalsis stopped once more when 5-hydroxytryptamine was washed out by flowing normal solution through the lumen. By adding a further dose of 4 μg. 5-hydroxytryptamine to the bath it was shown that the 5-hydroxytryptamine receptors in muscles or ganglia were still saturated. It was known from the results described in the first subsection of "Results" that this concentration of 5-hydroxytryptamine which caused the peristaltic block did not diminish the muscle response to the nervous transmitter acetylcholine. Therefore it was likely that peristalsis stopped as a result of ganglionic block. It could be restarted by stimulation of mucosal receptors with 5-hydroxytryptamine, or by stimulating the mucosal receptors with acetylcholine (Fig. 11*b*), or by serosal application of acetylcholine or nicotine.

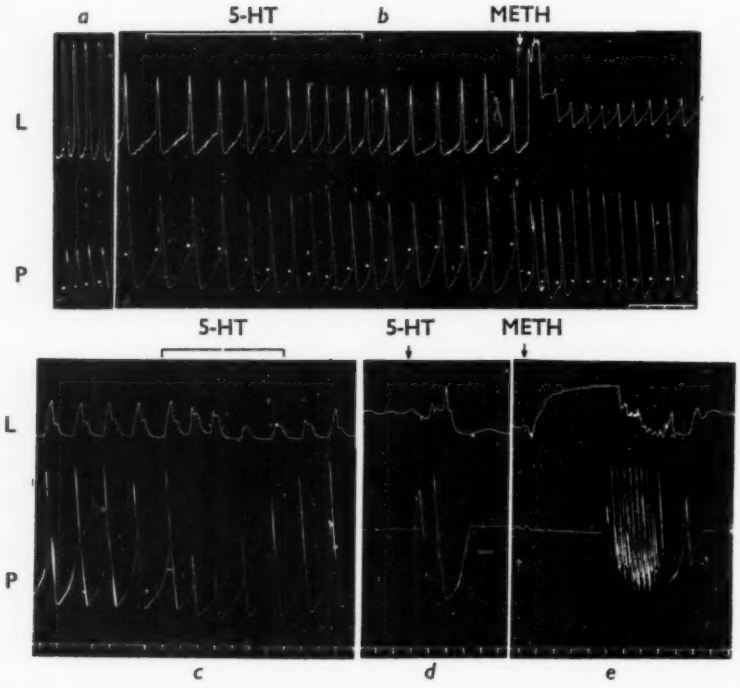

FIG. 10.—Records as in Fig. 5. Upper traces: (*a*) Normal peristalsis, (*b*) partial block in presence of $4{\cdot}8 \times 10^{-7}$ of atropine in bath. Effect produced by mucosal application (during the bracket) of 5-hydroxytryptamine (***5-HT***) 4×10^{-6}, and by addition (at the arrow) of 0·2 μg. of methacholine to the 50 ml. bath. (*c*) Partial block in the presence of 4×10^{-6} atropine in bath; effect of mucosal application (during bracket) of ***5-HT*** 4×10^{-6}, (*d*) complete block by atropine $1{\cdot}2 \times 10^{-5}$. Effect of serosal application (at arrow) of 4 μg. of ***5-HT***, (*e*) of 200 μg. methacholine at arrow. Time, 30 sec.

The Action of 5-Hydroxytryptamine on the Peristaltic Reflex in the Presence of Morphine, Phenoxybenzamine and Dihydroergotamine

Morphine was used to block 5-hydroxytryptamine receptors in nervous structures (Gaddum and Picarelli, 1957). When it was added to the fluid in the bath producing a concentration of 2 to 5×10^{-8}, morphine abolished the preparatory phase as well as the emptying phase immediately. However, while the longitudinal muscle contraction stopped completely, the circular muscle contracted at a very high frequency, but irregularly. When 5-hydroxytryptamine was introduced into the lumen these contractions became regular and soon reverted to co-ordinated peristaltic waves (Fig. 12*a*). The longitudinal contractions were only restored to some extent. Fig. 12 (*b* and *c*) shows that, when peristalsis was blocked by serosal application of morphine, serosal application of 5-hydroxytryptamine was ineffective at a time when mucosal application was still able to co-ordinate the high-frequency circular contractions and restore the reflex.

When morphine was added to the solution flowing through the lumen, 100 times higher concentrations (1 to 2×10^{-6}) were required to block the peristaltic reflex than when morphine was present in the bath. The effect, however, was the same as that of morphine acting from the serosal surface. The longitudinal contractions and the emptying phase were abolished, but the circular muscle showed irregular activity of high frequency. Mucosal application of 5-hydroxytryptamine again restored the reflex.

Phenoxybenzamine (Dibenzyline) and dihydroergotamine were used to block 5-hydroxytryptamine receptors in the muscles (Gaddum and Picarelli, 1957). Serosal application of phenoxybenzamine 3×10^{-7} or dihydroergotamine 3×10^{-6} stopped the peristaltic reflex, abolishing longitudinal as well as circular muscle contractions. 5-Hydroxytryptamine was effective in overcoming the block, but its action was much more powerful when it acted on the mucosa than when it acted from the serosal surface. In the presence of 3×10^{-7} of phenoxybenzamine, however, 5-hydroxytryptamine produced no recovery of longitudinal muscle contractions though it restored the peristaltic waves of the circular muscle. In some experiments it was observed that phenoxybenzamine caused a small contraction and some stimulation of peristalsis before it caused depression. This happened only when the intestine had already been treated with 5-hydroxytryptamine before phenoxybenzamine was given.

In order to block 5-hydroxytryptamine receptors in the muscles as well as in nervous structures, morphine (1×10^{-7}) and either phenoxybenzamine (1×10^{-7}) or dihydroergotamine (1.5×10^{-6}) were administered simultaneously. These concentrations caused at first partial block, but during prolonged exposure complete cessation of peristalsis. Fig. 13*a* and *b* illustrates one of these experiments. Phenoxybenzamine reduced the longitudinal contractions to about half and raised the threshold at which the reflex was elicited (*b*). Morphine abolished the longitudinal contractions but stimulated the circular muscle, which contracted irregularly and

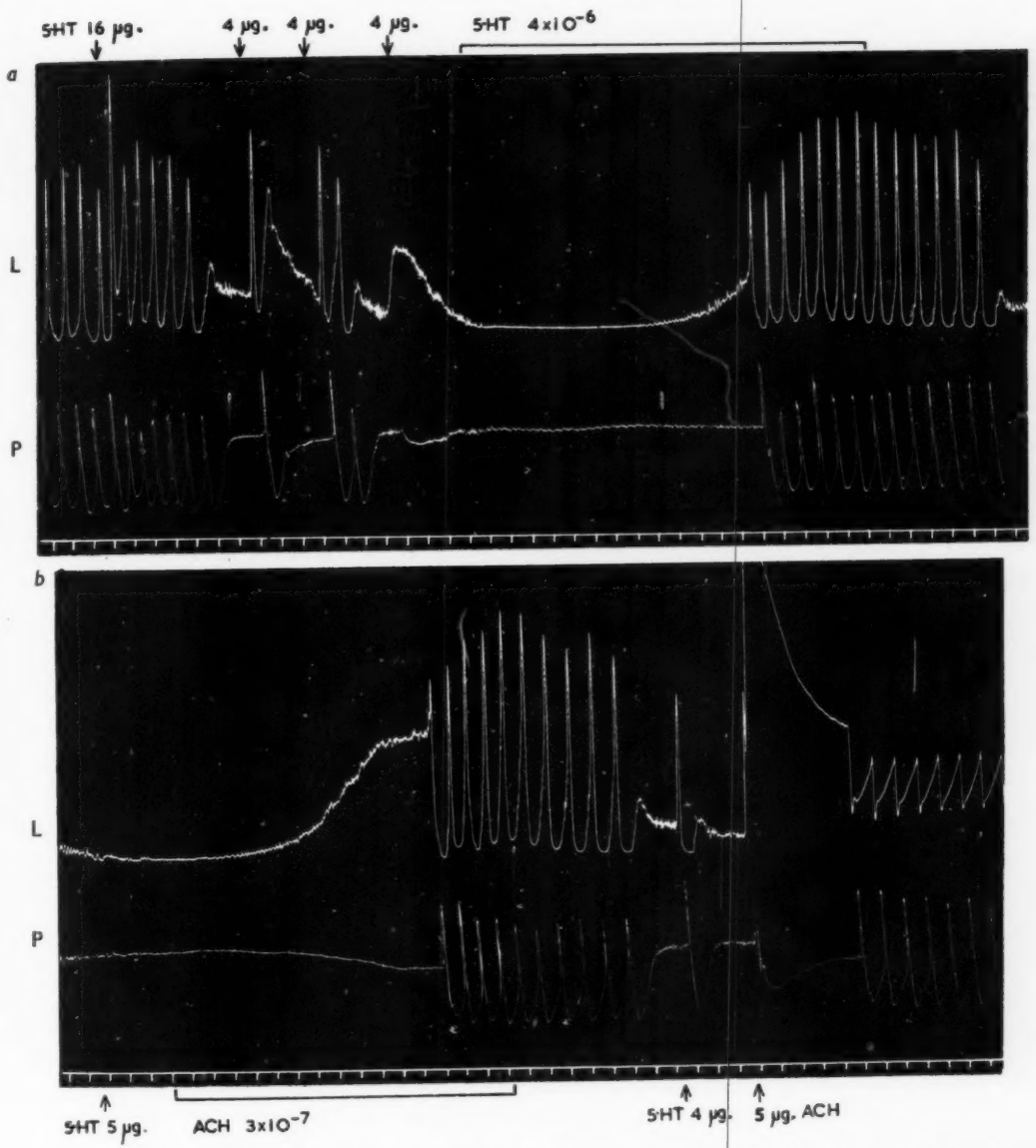

FIG. 11.—Records as in Fig. 5. (*a*) Peristalsis gradually blocked by repeated serosal application (at arrows) of small doses of 5-hydroxytryptamine (*5-HT*) and restored by mucosal application of *5-HT* (during bracket). (*b*) Continuation of record in (*a*), showing (during the bracket) effect of mucosal application of acetylcholine (*ACH*) 3×10^{-7}, and, at arrow, adding at 5 µg. *ACH* to 50 ml. bath. Time, 30 sec.

frequently. The introduction of 5-hydroxytryptamine into the intestinal lumen at once slowed the circular muscle movements, which now became co-ordinated; the longitudinal contractions also recovered to some extent. When 5-hydroxytryptamine was removed from the lumen peristalsis stopped. Serosal application of 5-hydroxytryptamine had no effect at this stage.

The most pronounced effect of 5-hydroxytryptamine, when introduced into the intestinal lumen and thus acting on the mucosa, was its co-ordinating action on the circular muscle contractions. Fig. 13*c* shows a tracing from an experiment in which peristalsis had been depressed by dihydroergotamine 1.5×10^{-6} in the bath. The addition of morphine 1×10^{-7} abolished longitudinal contractions and caused very frequent highly irregular movements of the circular muscle. When 5-hydroxytryptamine was added to the bath, it had no effect. However, for a short

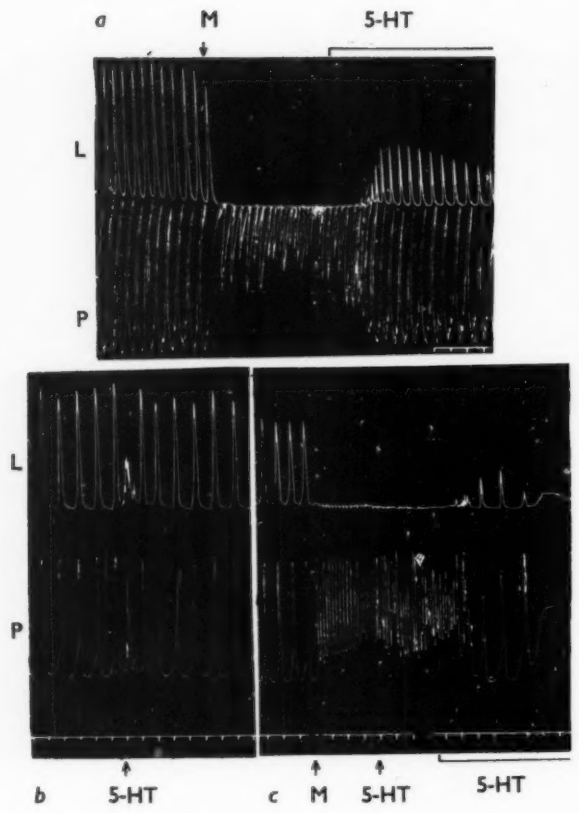

FIG. 12.—Records as in Fig. 5. (*a*) Abolition of peristalsis by adding 1 μg. morphine (*M*) to 50 ml. bath and restoration by presence of 5-hydroxytryptamine (*5-HT*) 4×10^{-6} in intestinal lumen (during the bracket). (*b*) and (*c*): traces from another experiment. (*b*) Effect of serosal application (at arrow) of *5-HT* (4 μg. in 50 ml.) on normal peristalsis. (*c*) Effect of 2·5 μg. morphine (*M*) after which serosal application (at arrow) of 4 μg. of *5-HT* had no action; but mucosal application (during the bracket) of *5-HT* 4×10^{-6} caused reappearance of co-ordinated waves. Time, 30 sec.

TABLE II

ACTION OF 5-HT ON PERISTALSIS IN PRESENCE OF 5-HYDROXYTRYPTAMINE ANTAGONISTS

Blocking Substance and Site of Application	Action of Blocking Substance on Longitudinal and Circular Muscle	5-Hydroxytryptamine: Serosal Application	5-Hydroxytryptamine: Mucosal Application	Comments on 5-Hydroxytryptamine Effect
5-Hydroxytryptamine Serosal	Longitudinal and circular contractions abolished Peristalsis abolished	0	+	Longitudinal and circular contractions restored Peristalsis restored
Morphine Serosal or mucosal	Longitudinal contractions abolished Circular contractions more frequent, irregular and uncoordinated Peristalsis abolished	0 0	+ +	Longitudinal contractions partly restored Circular contractions fully restored, slowed and co-ordinated Peristalsis restored
Phenoxybenzamine or dihydroergotamine Serosal	Longitudinal and circular contractions abolished Peristalsis abolished	(+) or 0	+	Longitudinal contractions partly restored Circular contractions fully restored Peristalsis restored
Morphine + phenoxybenzamine or dihydroergotamine Serosal	Longitudinal contractions abolished Circular contractions more frequent, irregular and uncoordinated Peristalsis abolished	(+) or 0	+	Longitudinal contractions partly restored Circular contractions fully restored, slowed and co-ordinated Peristalsis restored
2-Bromo-lysergic acid diethylamide or lysergic acid diethylamide Serosal	Longitudinal contractions continue Circular contractions abolished Peristalsis abolished	0	0	No effect

period during which 5-hydroxytryptamine was present in the intestinal lumen, regular peristalsis was restored; and this reverted to the irregular high-frequency activity as soon as the 5-hydroxytryptamine was washed out from the lumen.

These experiments, which are summarized in Table II, show that mucosal application of 5-hydroxytryptamine was always effective when 5-hydroxytryptamine receptors had been blocked selectively at nervous structures in the outer wall by morphine, or selectively at the muscle by phenoxybenzamine or dihydroergotamine, or when both kinds of receptors had been blocked. Serosal application in these conditions produced either no effect or a much smaller one than that on the mucosa. These results lent further support to the view that the two sites of action of 5-hydroxytryptamine were different and that serosal application did not reach mucosal sensory receptors; nor did mucosal application reach muscle or nervous structures in the outer wall.

The Effect of 2-Bromo-lysergic Acid Diethylamide and Lysergic Acid Diethylamide

When 2-bromo-lysergic acid diethylamide 8×10^{-6} or lysergic acid diethylamide 1.2×10^{-5} was present in the bath the peristaltic reflex was abolished (see Kosterlitz and Robinson, 1957; Ginzel, 1957). It was noted, however, that longitudinal muscle contractions still occurred at regular intervals, as shown in Fig. 14. In these conditions 5-hydroxytryptamine was entirely without action both from the serosal and the mucosal surface.

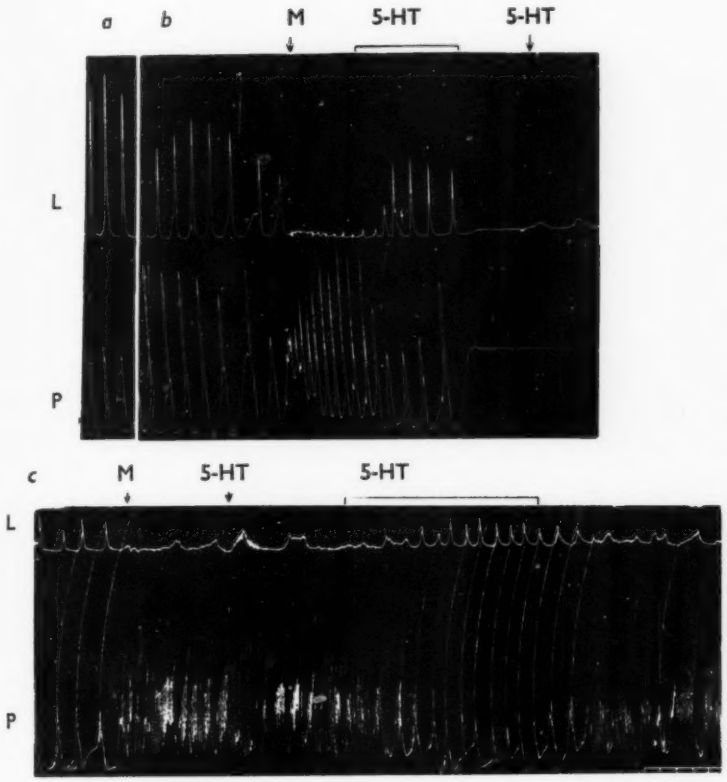

FIG. 13.—Records as Fig. 5. (*a*) before, (*b*) in the presence of phenoxybenzamine 10^{-7} in bath. Morphine 10^{-7} at *M*. Mucosal 5-hydroxytryptamine (*5-HT*) during bracket and serosal *5-HT* at arrow. (*c*) Trace from another experiment in presence of dihydroergotamine $1{\cdot}5 \times 10^{-6}$ in bath. Morphine 10^{-7} at *M*. Serosal *5-HT* at arrow and mucosal *5-HT* during bracket. Time, 30 sec.

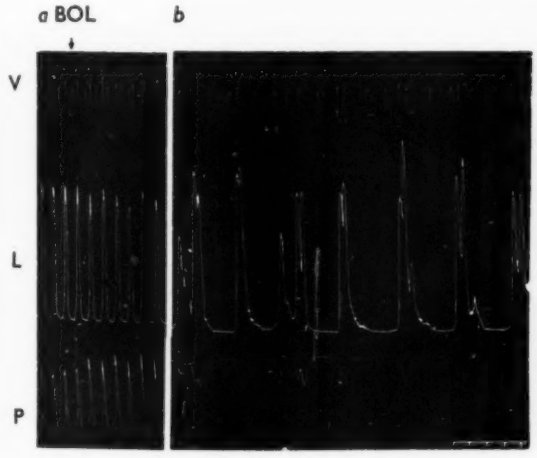

FIG. 14.—Records as in Fig. 5. Effect of serosal application of 2-bromo-lysergic acid diethylamide (BOL). (*a*) Immediate effect of 7×10^{-7} of BOL at arrow. (*b*) Final condition in presence of 8×10^{-6} of BOL in the bath. Time, 30 sec

DISCUSSION

When an organ of such complicated and only incompletely known anatomy as that of the intestine is exposed simultaneously to several pharmacologically active substances, phenomena are produced which are difficult to analyse. Nevertheless the present work has brought out several points which may clarify the action of 5-hydroxytryptamine.

The motor transmitter to intestinal muscle is generally believed to be acetylcholine, and supporting evidence for this view has recently been put forward by Paton (1956). In the present work it was found that 5-hydroxytryptamine, in addition to its own stimulant action, sensitized intestinal muscle to the action of acetylcholine. Moreover, at a time when 5-hydroxytryptamine receptors in the muscle were insensitive to 5-hydroxytryptamine after saturation with 5-hydroxytryptamine itself, the muscle was still hypersensitive to acetylcholine. The short-lasting facilitation of the peristaltic reflex which was observed after serosal application of 5-hydroxytryptamine might therefore be explained by sensitization at muscular sites.

This action of 5-hydroxytryptamine was similar to that in the sympathetic superior cervical ganglion described by Trendelenburg (1956a and b). He found that 5-hydroxytryptamine stimulated the ganglia, facilitated but never blocked ganglionic transmission, and sensitized the ganglia to injected acetylcholine which is the synaptic transmitter substance. The nature of the synaptic transmitter from sensory to motor neurons in the gut is not known. Marrazzi (1953) observed that 5-hydroxytryptamine blocked synapses in the central nervous system. All the evidence which we obtained indicated that in intestinal ganglia 5-hydroxytryptamine caused a transient facilitation of transmission followed by block. This assumption was based on the following observations:

While the longitudinal contractions of an isolated piece of intestine in response to acetylcholine were never depressed by 5-hydroxytryptamine, the nicotine response, after a transient increase, was always depressed by 5-hydroxy-

tryptamine. This indicated that 5-hydroxytryptamine depressed ganglionic excitability.

The peristaltic reflex was blocked by serosal application of 5-hydroxytryptamine. This could be overcome by serosal application of nicotine or acetylcholine. Moreover, when the peristaltic reflex was blocked by serosal application of 5-hydroxytryptamine itself, the block could also be overcome by applying 5-hydroxytryptamine to the mucosa. This last observation was of particular significance because it showed that 5-hydroxytryptamine acted on different sites according to the site of application. When it was added to the fluid in the bath it caused ganglion block. When it was introduced into the lumen it reinforced the sensory impulses arising from the mucosa and re-established the reflex pathway.

Recently Beleslin and Varagić (1958) showed that, when the peristaltic reflex was abolished by cooling the intestine, not only mucosal but also serosal application of 5-hydroxytryptamine restored the reflex. The authors believed that, when ganglionic transmission had been depressed by cooling, it could be stimulated by 5-hydroxytryptamine acting from the serosa and, though low concentrations facilitated transmission, high concentrations produced ganglion block. Furthermore, when in the cooled intestine block was produced after serosal application of a large dose of 5-HT, it was possible, by introducing the drug into the lumen, to overcome the block. In the present investigation results similar to those of Beleslin and Varagić (1958) were obtained at normal temperatures.

The action of some other sensory stimulants which were studied was similar to that of 5-hydroxytryptamine. When tested on the longitudinal muscle contractions in a piece of intestine, they had no depressant effect on the response to acetylcholine but they all depressed the response to nicotine. They all depressed the peristaltic reflex when applied to the serosal surface of a loop of intestine, and they all facilitated the peristaltic reflex when applied to the mucosa.

The experiments in which the peristaltic reflex was blocked by various substances acting specifically on different parts of the reflex arc threw further light on the various sites of action of 5-hydroxytryptamine.

When mucosal sensory receptors had been blocked by introducing procaine into the lumen of the intestine, 5-hydroxytryptamine applied to the mucosa was effective, but 5-hydroxytryptamine applied to the serosa was ineffective in overcoming the block. Thus when the sensory stimuli arising from the mucosa were diminished or absent, no facilitation at ganglionic or muscular sites by 5-hydroxytryptamine could restore peristalsis, but sensory stimulation at the mucosa by 5-hydroxytryptamine re-established the reflex; so did phenyldiguanide.

When ganglionic or neuromuscular transmission had been impaired by hexamethonium or atropine, the facilitation at ganglionic or muscular sites by 5-hydroxytryptamine as well as the reinforcement by 5-hydroxytryptamine of sensory stimuli arising from the mucosa re-established the reflex pathway. However, during complete block by hexamethonium or atropine, 5-hydroxytryptamine applied to the mucosa was less effective when applied to the serosa.

A few experiments were carried out using dihydroergotamine or phenoxybenzamine to block selectively muscular 5-hydroxytryptamine receptors and morphine to block nervous 5-hydroxytryptamine receptors (Gaddum and Picarelli, 1957). Morphine abolished the preparatory as well as the emptying phase (Kosterlitz and Robinson, 1955; Schaumann, 1955) but stimulated the circular muscle to high-frequency activity, which, however, did not lead to fluid propulsion. In this condition mucosal application of 5-hydroxytryptamine had a striking effect: it slowed the excessive activity and transformed the contractions of the circular muscle into well-coordinated peristaltic waves.

It has been shown that morphine diminishes the release of acetylcholine from postganglionic motor nerve endings (Paton, 1957; Schaumann, 1957), which might be part of its blocking action on peristalsis. In these conditions serosal application of 5-hydroxytryptamine can have no effect because morphine blocks its action. If morphine diminished the release of the preganglionic sensory transmitter, the mucosal application of 5-hydroxytryptamine might be capable of re-establishing the reflex pathway through stimulation of mucosal sensory receptors, as in fact it does.

Procaine has been shown by Harvey (1939) to diminish the release of acetylcholine at preganglionic nerve endings. In this connexion it should be pointed out that the action of morphine in stimulating high-frequency activity in the circular muscle was not dissimilar from the effect seen in the presence of local anaesthetics. Here also, 5-hydroxytryptamine applied to the mucosa facilitated the peristaltic reflex, but applied to the serosa it had no effect.

The origin of the circular muscle activity produced by morphine is not explained. Increased muscle tone has been observed by many authors after administration of morphine. The mechanism has been discussed by Vaughan Williams and Streeten (1950), who pointed out that it might be a muscular response when the intestine is kept forcibly dilated. In the experimental conditions of the present investigation the intraluminal pressure remained high unless the fluid was expelled by a wave of peristalsis. It may be that the circular contractions represent the muscular response to the prolonged distension of the wall, which occurs when the nervous control, which normally co-ordinates the contractions to propulsive waves, has been removed by morphine. On the other hand, it may be the result of a direct stimulant action of morphine on the smooth muscle which has been observed by Bülbring and Burnstock (unpublished observations).

This work has been done during the tenure by one of us (A. C.) of a Riker Fellowship for which he wishes to express his thanks to the Riker Laboratories, Ltd. We are indebted to Professor O. Krayer for the samples of pure veratrine and protoveratrine to Smith, Kline and French for the phenoxybenzamine, and to Sandoz, Ltd., for 5-hydroxytryptamine creatinine sulphate.

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CHEMOTHERAPEUTIC SCREENING OF COMPOUNDS AGAINST *THEILERIA ANNULATA* IN TISSUE CULTURE

BY

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Theileria annulata has been grown in tissue culture and these cultures have been used for screening compounds for chemotherapeutic activity against this parasite. The method described is simple and convenient for testing many compounds in a short time. Forty compounds, including antimalarials, trypanocides, and antibiotics, have been examined, but none has been found to be active.

A technique is described for screening of compounds for therapeutic activity against *Theileria annulata* growing in tissue culture. Preliminary results with compounds are reported ; no activity was discovered in the present series.

METHODS

Tissue samples of lymph nodes, spleen, and liver containing *Theileria annulata* were kindly sent to me by air by Dr. I. Tsur of the Veterinary Institute, Tel Aviv, Israel. They were packed in tubes containing Hanks' or Earle's solution (both seemed equally good). When smeared, the tissues usually showed 1 to 10 Koch bodies/oil immersion microscope field. On arrival at this laboratory, they were washed in a solution containing penicillin (100 units/ml.) and streptomycin (100 units/ml.). The tissue was then cut into implants which were set up in Carrel flasks by the technique decribed by Brocklesby and Hawking (1958) which was adapted from that originally described by Tsur (Tchernomoretz), 1945. Briefly implants were fastened by a plasma clot to small coverslips (0.7 $cm.^2$) glued to the floor of a Carrel flask with plasma. The fluid medium consisted either of calf serum 40%, embryo extract 2.5%, and medium 199 57.5% (Morton, Morgan, and Parker, 1956) ; or of calf serum 40%, embryo extract 2.5%, tyrode 57%, phenol red to colour, and 0.5% of a solution containing glutamine 60 mg., pyridoxine 12 mg., pyridoxal 10 mg., riboflavin 2 mg., inositol 80 mg., water 100 ml. The latter medium is referred to here as Tsur's medium, but in fact differs from Tsur's original medium in some minor details and in that it contains embryo extract. Both media contained penicillin 50 units/ml. and streptomycin 50 units/ml.

Growth of cells and of parasites was somewhat irregular, but there was no constant difference between the two media. Spleen tissue grew more vigorously than lymph node, but lymph node, which usually contained more parasites initially, often gave a better growth of parasites ; implants of liver did not yield significant growth. After 2 to 4 days, when it was established that growth of cells was taking place, the cultures were prepared for chemotherapeutic testing in two alternative ways. In one method, the coverslips carrying the cultures had been set up in large Carrel flasks (8 cm. diameter) each containing 20 slips. In this case the slips were removed and were placed individually in test tubes (15 ml.) each containing 2 ml. of fresh medium. In the other method, the slips were set up in small flasks (3 cm. diameter), each flask containing 3 slips ; in this case the old medium was removed and 3 ml. of new medium was inserted. Drugs dissolved in Ringer solution were then added to the tubes or small flasks (0.1 ml. to 2 ml. or 0.15 ml. to 3 ml.). The concentrations of drugs were arranged in steps of $\times 3.2(\sqrt{10})$. The tubes were placed on a roller machine at 36° and the flasks were incubated at the same temperature. After 5 to 6 days slips were removed and prepared for examination. The exact times in the above scheme can be varied according to experience and to the acidity of the medium ; if the flasks became yellow too soon, 1 to 4 drops of 1.3% sodium bicarbonate were added to neutralize the acidity. Probably the best results were obtained with a preliminary period of 2 days and exposure to drug for 5 days.

For examination, the slip was removed from the tube or flask. The centre of the implant was picked off with fine forceps and dabbed on a slide to make a series of small smears. This slide was later dried, fixed in alcohol, and stained with Giemsa. The slip with the outgrowth from the implant was dropped into methyl alcohol for fixation, stained with Giemsa, dehydrated by drying in the incubator, and mounted culture downwards with any of the customary mountants. The growth of the parasites, which varied considerably even in the control tubes without

drug, was patchy and unevenly distributed. Some smears showed five or more parasites (Koch bodies)/oil immersion field; others showed only one or two parasites during a 10 min. search. Examination of the smears was usually more rewarding than examination of the cultures fixed *in situ*; parasites were difficult to find in the cultures unless many were present. On the other hand, the cultures were better than the smears for showing the reaction of the cells to the drug. In view of this uneven growth, it is necessary to examine each concentration of drug several times or in several experiments. If well-preserved parasites were discovered, it was concluded that the drug had not destroyed them. If no parasites were discovered, although the cells seemed relatively healthy no conclusion was drawn. The finding of healthy parasites in two separate experiments was considered evidence that the drug failed to act. Demonstration of an active compound (which did not occur during the present work) would depend upon the consistent absence of parasites from cells which remained healthy.

TABLE I

COMPOUNDS TESTED

The values in this table give an approximate estimation of the maximum concentration tolerated by lymphocytes and macrophages.

Compound	mg./100 ml.
Antimalarials	
Quinine bisulphate (1 expt.)	?1
Pamaquin	0·01–0·03
Pentaquin diphosphate	0·05
Rhodoquin dihydrochloride	0·03
Chloroquine phosphate	0·03
Sontoquin sulphate (1 expt.)	0·03–0·1
Mepacrine hydrochloride	0·2
Pyrimethamine	3–10
Diamidines	
Stilbamidine nitrate	0·3–1
Berenil	1
Sulphonamides, etc.	
Sulphadiazine	5
Dapsone	3
Maphenide (Marfanil)	3
Metachloridine, 2-(*m*-aminobenzenesulphonamido)-5-chloropyrimidine	3
Arsenicals	
Melarsoprol (Mel B; Arsobal)	0·1
Arsenamide sodium, p-NH_2.CO.C_6H_4.As(S.CH_2.$CO_2Na)_2$ (Otto and Maren, 1947; 1950)	0·1
Antimonials	
Stibophen	0·1
Antimony potassium tartrate (tartar emetic)	0·1
Trypanocides	
Suramin	5
Quinapyramine (Antrycide)	1
Prothidium (R.D. 2801) (Watkins and Woolfe, 1956)	0·1–0·3
Styryl 314, 2-(*p*-acetamidostyryl)-6-dimethylamino-quinoline methochloride (1 expt.)	?0·3
Bayer 7602 (Fulton, 1943)	1
1-Methyl-1-phenyldithiobiuret (RD 1660) (Woolfe, 1953)	1
Miscellaneous	
Lucanthone hydrochloride	0·1–0·3
Cyanine 863, 1′-ethyl-3 : 6-dimethyl-2-phenyl-4-pyrimidinyl-2′-cyanine (Welch, Peters, Bueding, Valk and Higash, 1947)	0·1–0·3
Methylene violet, 3-amino-7-dimethylaminophenosafranine (Hawking, Ormerod, Thurston and Webber, 1952)	0·1–0·3
Isoniazid	10–30
BIQ 20, eicosamethylenebis(*iso*quinolinium iodide) (Austin, Lunts, Potter and Taylor, 1959) (1 expt.)	0·1–0·3
BIQ 12, dodecamethylenebis(*iso*quinolinium iodide)	0·1
BAC 20, eicosamethylenebis(4-aminocinnolinium iodide)	0·1
3 : 4-Dimethyl-ω-piperidinoacetanilide hydrochloride (W 2)	1–3
1–(3 : 4-Xylylcarbamoylmethyl)pyridinium chloride (W 6)	3–10
N-β-Piperidinoethyl-3 : 4-xylidine dihydrochloride (W 55) (1 expt.)	3
Antibiotics	
Chlortetracycline (aureomycin)	10
Chloramphenicol	10
Neomycin	3
Viomycin sulphate	10
Bacitracin	10
Erythromycin (insoluble)	?10
Vulcamycin (insoluble)	?10

RESULTS

To test the screening technique and to make a preliminary survey of the chemotherapeutic reactions of *T. annulata*, a series of compounds was tested (Table I). A range of concentrations of each drug was employed, the highest concentration being sufficient to kill or severely injure the host cells. The values given in Table I give an estimate of the maximum concentration (mg./100 ml.) tolerated by the cells (lymphocytes and macrophages) without great degeneration. None of the 40 compounds showed activity in preventing the survival of Koch bodies for 5 to 6 days. In view of reports suggesting possible activity in cattle, particular attention was paid to aureomycin, to the 8-aminoquinoline compounds (pamaquin, pentaquine and Rhodoquin) and to mepacrine, but these compounds were not effective in our cultures. Penicillin and streptomycin (which were present in all the culture media) did not prevent growth of parasites.

DISCUSSION

The above technique provides a method for laboratory screening of compounds for chemotherapeutic activity upon *Theileria annulata*. Provided a source of infected tissue is available, it is simple and convenient; over a hundred drug concentrations or duplicates can be easily examined in a single experiment. Like all *in vitro* tests, it might fail to indicate compounds the activity of which depended upon co-operation of the tissues of the host; but, since some host cells are present in the cultures, these might be sufficient for many of such compounds. Theoretically, the activity of some compounds might depend upon an antimetabolite effect; accordingly the sensitivity of the test would probably be improved if a medium less rich in growth stimulants than medium 199 was used; for example, Tsur's medium or simple Tyrode solution. The method examines for activity against *T. annulata*, the Middle East species of theileria. It is not known

whether *T. parva,* the East African parasite, differs from *T. annulata* in its chemotherapeutic reactions since no highly effective drug is yet known for either of them. Growth of *T. parva* in tissue culture is still unsatisfactory (Tsur, Neitz and Pols, 1957 ; Brocklesby and Hawking, 1958) ; until this position has been remedied, *T. annulata* would appear to be the best organism for laboratory tests. *T. mutans* (English strain) has been examined in our laboratory, but no Koch bodies could ever be found by gland puncture.

The experimental results described above failed to reveal therapeutic activity in any of the 40 compounds studied. This agrees broadly with experience in the field ; although many compounds have been claimed to be curative for *T. annulata,* none of these claims has received general acceptance. The number of compounds tested so far on this infection is still, however, small, and further systematic screening might be expected to reveal activity in due course.

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THE ACTION OF SYMPATHOMIMETIC AMINES ON HEART RATE IN RELATION TO THE EFFECT OF RESERPINE

BY

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When the heart-lung preparation is made from a dog treated with reserpine, catechol amines such as noradrenaline and isoprenaline have a greater effect on the rate of the heart than they have in a preparation from a normal dog. Other sympathomimetic amines such as tyramine and ephedrine, on the other hand, are found to have lost their action. Since treatment with reserpine has been shown to cause the store of noradrenaline in the heart to disappear, and the infusion of noradrenaline into the preparation made from a reserpine-treated animal restores the action of tyramine, it is concluded that substances like tyramine and ephedrine normally act by liberating noradrenaline from the store, and do not act directly. Cocaine, like reserpine, increases the effect of noradrenaline and decreases the effect of tyramine on the heart rate; it appears to block the release of noradrenaline from the store in the heart.

Observations have recently been made (Burn and Rand, 1958c) on the action of sympathomimetic amines on the blood pressure of the spinal cat and on the vessels of the perfused hindleg of the dog. Experiments were performed on normal animals, and on animals treated with reserpine. In animals treated with reserpine the effect of the catechol amines such as noradrenaline, adrenaline and dopamine was greater than usual, the tissues being supersensitive to them. However, the effect of other amines such as tyramine and ephedrine was almost abolished. Since it had been shown (Burn and Rand, 1957, 1958b) that reserpine causes the dispersal of the noradrenaline in the vessel wall, the conclusion was drawn that tyramine and similar amines act by releasing noradrenaline, and that they differ from the catechol amines which act directly.

We now describe a study of the action of sympathomimetic amines on the heart rate.

METHOD

Observations were made in the heart-lung preparation of the dog, at a temperature between 36.5° and 37°. One dog was bled under ether anaesthesia. The second dog was first anaesthetized with ether and chloralose was then injected intravenously. The heart-lung preparation was then made. The venous reservoir was immersed in a thermostatically controlled water bath, with an overflow from the reservoir so that the venous pressure was constant. The heart rate was measured from the electrocardiogram recorded on a Cossor electrocardiogaph model 1314. The artificial resistance was set to maintain a pressure of 110 mm. and the systemic outflow was usually about 750 ml./min. Dogs usually weighed 12 to 15 kg. Those treated with reserpine were given 5 mg. on two successive days by intraperitoneal injection and were used on the third day. The solution of reserpine was prepared in 20% (w/v) ascorbic acid.

RESULTS

Tyramine and Phenylethylamine.—Tyramine hydrochloride was tested, as were the other amines, by injecting a dose into the tube carrying blood to the superior vena cava and observing the height and duration of the rise in heart rate which

TABLE I

EFFECT OF TYRAMINE AND PHENYLETHYLAMINE ON HEART RATE IN THE DOG HEART-LUNG PREPARATION

Substance	Dose (mg.)	Untreated		Reserpine-treated	
		Increase in Rate (Beats/Min.)	Duration of Effect (Min.)	Increase in Rate (Beats/Min.)	Duration of Effect (Min.)
Tyramine ..	0·4	25	19	2	4
	0·4	21	20	—	—
	0·8	—	—	2	3
	1·0	86	36	—	—
	1·0	55	41	—	—
	1·0	82	34	—	—
	2·0	—	—	25	22
	2·0	—	—	7	13
Phenyl-ethylamine	0·4	11	2	—	—
	1·0	67	14	—	—
	1·0	76	8	—	—
	1·0	50	6	—	—
	2·0	—	—	34	11
	2·0	—	—	10	4

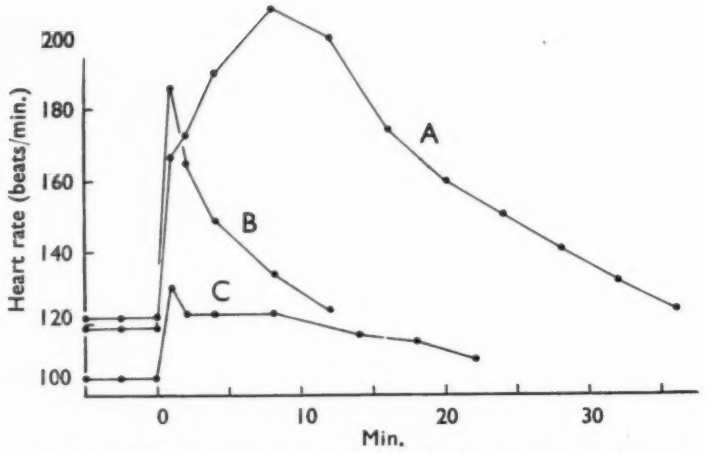

FIG. 1.—Dog heart-lung preparation. The effect upon the heart rate of injection at time 0 min. of 1 mg. of tyramine (A) and 1 mg. of phenylethylamine hydrochloride (B). The effect of 2 mg. of tyramine in a dog treated with reserpine is shown at C.

followed. As the example in Fig. 1 shows, the rise did not reach its maximum until some minutes after the injection, and it lasted for 30 to 40 min. when the dose was 1 mg. The results of observations in different experiments are shown in Table I. When the dog was treated with reserpine beforehand, the effect of tyramine was greatly reduced. The largest effect is shown in Fig. 1 **C**, and was made after the injection of 2 mg.

The rise produced by phenylethylamine differed in all observations from that produced by tyramine in reaching its maximum point in 1 min., and then rapidly declining so that the effect was finished in 10 min. The action of phenylethylamine also was much smaller in dogs treated with reserpine.

Amphetamine. — The injection of amphetamine was followed by a rise in heart rate which seemed to persist indefinitely. How much more prolonged it was in comparison with the rise due to tyramine can be seen in Fig. 2, in which an injection of 0.4 mg. of tyramine (**A**) was followed by an injection of 0.4 mg. of amphetamine (**B**). The rise caused by tyramine was over in 19 min. while the rise caused by amphetamine was still half its maximum value after 2.5 hr. In another experiment an injection of only 0.1 mg. of amphetamine caused a rise which was still half its maximum value after 1 hr. In a reserpine-treated dog, the injection of 0.4 mg. of amphetamine caused only a trace of effect on the heart rate as shown in Fig. 2 **C**.

The Phenylethanolamines. — We were able to test both optical isomers of phenylethanolamine, having samples prepared by Professor P. Pratesi of Pavia (Pratesi and Grassi, 1953). When they were compared in a normal preparation, they differed in duration of action, the effect of (−)-phenylethanolamine passing off more quickly as shown in Fig. 3. Burn and Rand (1958c) observed a similar difference on the blood pressure. In a preparation from a reserpine-treated dog, the effect of both was greatly reduced, though the injection of (−)-phenylethanolamine produced a somewhat greater effect than the other isomer, and this again recalled a similar difference on the blood pressure.

Ephedrine.—Ephedrine is known to produce a prolonged rise in heart rate (Krayer and Ourisson, 1954). Axelrod (1953) has shown that it is converted in the body into norephedrine, which is the active substance and which differs only from (−)-phenylethanolamine in having a methyl group on the α carbon atom. The relation between these two substances is therefore the same as the relation between amphetamine and phenylethylamine. The prolonged action of ephedrine and of amphetamine can therefore be ascribed to the presence of this methyl group which interferes with their destruction by amine oxidase. Fig. 4

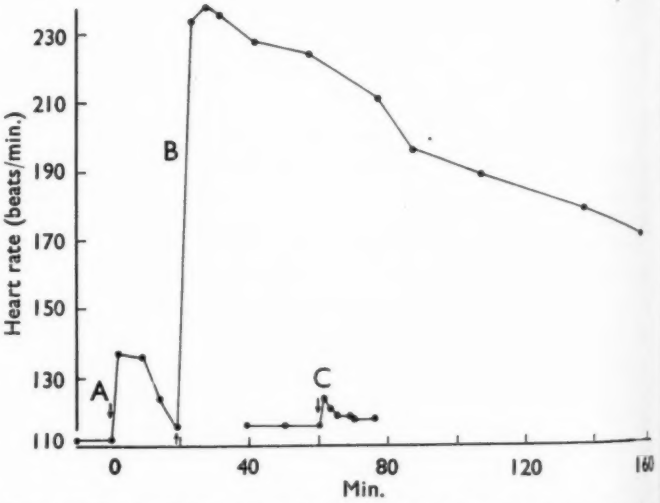

FIG. 2.—Dog heart-lung preparation. The effect upon the heart rate of a preparation made from an untreated dog of injection (at arrows) of 0.4 mg. of tyramine (A) and 0.4 mg. of amphetamine (B). The response to 0.4 mg. of amphetamine in a preparation made from a dog previously treated with reserpine is shown by curve C.

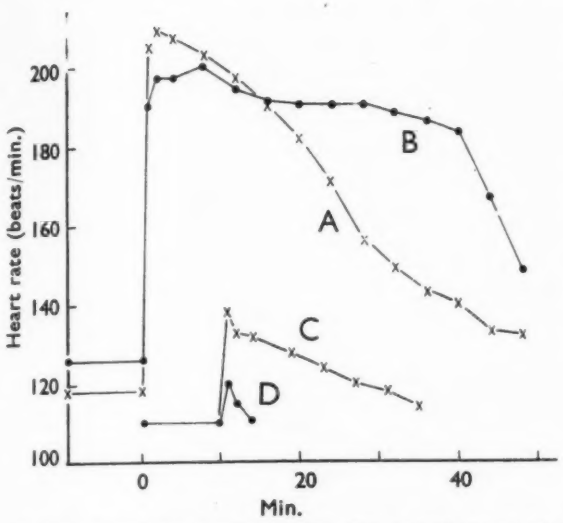

FIG. 3.—Dog heart-lung preparation. The effect on the heart rate of a preparation made from an untreated dog of 2 mg. of (−)-phenylethanolamine (A) and 2 mg. of (+)-phenylethanolamine (B). The responses of a preparation made from a dog previously treated with reserpine to 2 mg. of (−)-phenylethanolamine and 2 mg. of (+)-phenylethanolamine are shown by curves C and D, respectively.

shows a comparison between the effect on the heart rate of 0.4 mg. of (−)-phenylethanolamine and that of 0.4 mg. of ephedrine which was given immediately after. The effect of ephedrine was still half the maximal value after 2 hr. In the dog treated with reserpine, the injection of 1 mg. of ephedrine had no appreciable effect (Fig. 4 **C**).

Infusion of Noradrenaline.—When a cat was treated with reserpine, the injection of tyramine into the spinal preparation caused little or no rise of blood pressure. If, however, a slow intravenous infusion of noradrenaline was given during a period of 15 or 20 min., the total amount infused being from 0.5 to 1.0 mg., then when the infusion was stopped and the blood pressure had returned to the level at which it was before the infusion began, the injection of tyramine caused a rise of blood pressure (Burn and Rand, 1958c). We carried out experiments to see if a similar effect was to be observed in the heart-lung preparation. Having first established that tyramine had little effect on the heart rate of a preparation from a reserpine-treated dog, we infused noradrenaline slowly into the superior vena cava until a given amount had entered the blood. The infusion caused a rise in heart rate, and when the infusion was complete we waited until the heart rate returned to the rate at which it was before the infusion began. We then determined the effect of the same dose of tyramine again. In one experiment, the injection of 0.4 mg. of tyramine caused a rise of only 15 beats/min. The infusion of 2 mg. of noradrenaline was then made during 18 min. The rate returned to the pre-infusion level after 1.75 hr. The injection of 0.4 mg. of tyramine then caused a rise of 53 beats/min.

In the experiment illustrated in Fig. 5, smaller amounts of noradrenaline were infused, and the initial rise in rate was not affected. However, the duration of the effect of injecting 0.4 mg. of tyramine was increased by a first infusion of 0.25 mg. of noradrenaline (Fig. 5 curve **B**) and was still further increased by a second infusion of 0.5 mg. of noradrenaline (Fig. 5 curve **C**).

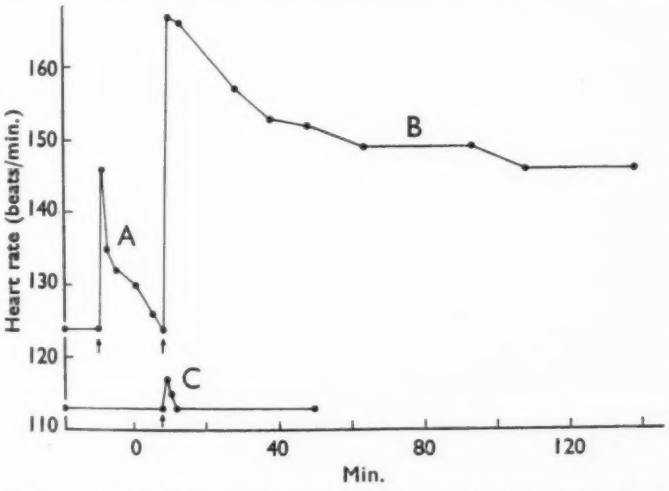

FIG. 4.—Dog heart-lung preparation. The effect on the heart rate of a preparation made from an untreated dog of 0.4 mg. of (−)-phenylethanolamine (A) and 0.4 mg. of ephedrine (B). The response in a preparation made from a dog previously treated with reserpine to 1 mg. of ephedrine is shown by curve C.

Catechol Amines.—Though the pressor effect of tyramine and related amines was very small in the reserpine-treated cat, the pressor effect of the catechol amines was very large. Comparisons were therefore made between the action of noradrenaline, dopamine and isoprenaline in the heart-lung preparation from an untreated dog and their action in the preparation from a reserpine-treated dog. The action of noradrenaline and isoprenaline was greater in the reserpine-treated preparation, and the action of dopamine was reduced. The details of all observations are given in Table II, which shows that the greater effect of noradrenaline was always seen. An example is shown on the right of Fig. 6. In observations on the blood pressure the greater effect of noradrenaline in the reserpine-treated cat was reduced after an intravenous infusion

of noradrenaline. This was also true in the heart-lung preparation. Thus the rise of 74 beats/min. caused by the injection of 4 μg. of noradrenaline in a reserpine-treated preparation was reduced after an infusion of noradrenaline to a rise of only 8 beats/min. Similarly the rise of 42 beats/min. produced in another experiment was reduced by an infusion of noradrenaline to a rise of only 18 beats/min.

TABLE II

INCREASE IN HEART RATE CAUSED BY CATECHOL AMINES IN THE DOG HEART-LUNG PREPARATION

Substance	Dose	Normal		Reserpine	
		Increase (Beats/Min.)	Mean	Increase (Beats/ Min.)	Mean
Noradrenaline ..	1 μg.	0, 5, 9	4·7	11, 25, 18	18·0
,, ..	2 μg.	6, 10, 16, 14	11·5	18, 19, 29	22·0
,, ..	4 μg.	15, 31, 22	22·7	74, 42	58·0
Dopamine ..	0·1 mg.	19, 10	14·5	3, 7, 3	4·3
,, ..	0 2 mg.	57, 23	40·0	5, 18, 10	11·0
Isoprenaline ..	0·5 μg.	20		23	
,, ..	2 μg.	53		80	

While the action of dopamine on the vessels resembled that of noradrenaline, being increased in the reserpine-treated preparation, this was not true in the heart-lung preparation. The effect of dopamine was less, as shown in Table II. Moreover after an infusion of noradrenaline the action of dopamine was increased. Thus the injection of 0.1 mg. of dopamine caused a rise of only 7 beats/min. in one reserpine-treated preparation. After an infusion of noradrenaline, 0.1 mg. of dopamine caused a rise of 32 beats/min. Similarly in a second preparation the injection of 0.2 mg. of dopamine caused a rise of 10 beats/min., but after an infusion of noradrenaline it caused a rise of 22 beats/min.

The evidence concerning isoprenaline depended on only four observations. However, the duration of action was greater in the reserpine-treated animal. Thus, 0.5 μg. of isoprenaline caused a rise of 20 beats/min. in a normal preparation, which passed off in 4 min., and in a reserpine-treated preparation it caused a rise of 23 beats/min. which lasted for 20 min. Similarly, 2 μg. of isoprenaline caused a rise of 53 beats/min. which was finished in 12 min. in a normal preparation, but it caused a rise of 80 beats/min. which was not finished by 30 min. in a reserpine-treated preparation.

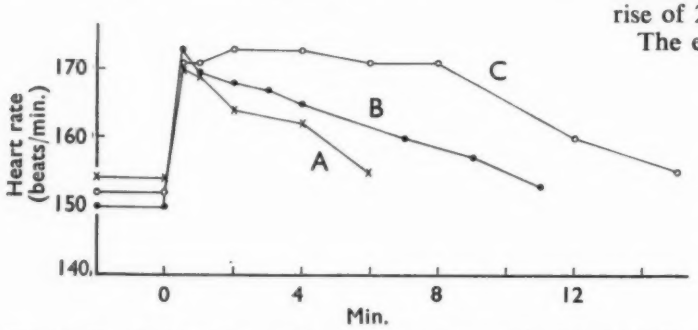

FIG. 5.—Dog heart-lung preparation made from a dog previously treated with reserpine. (A) The response of the heart rate to 0.4 mg. of tyramine; (B) the response to 0.4 mg. of tyramine after an infusion of 0.25 mg. of noradrenaline; (C) the response to 0.4 mg. of tyramine after infusion of a further 0.5 mg. of noradrenaline.

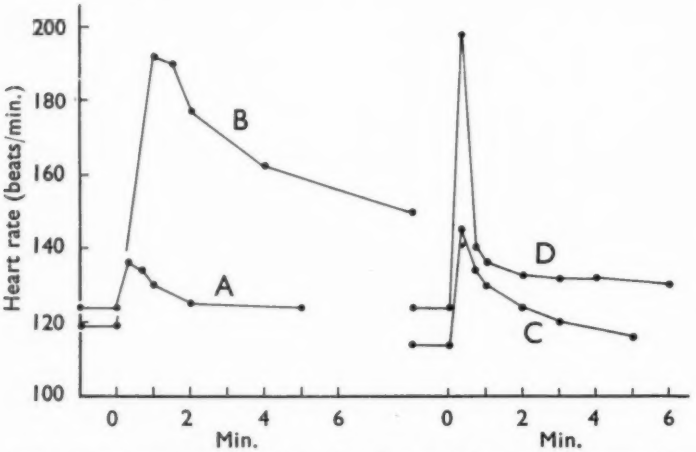

FIG. 6.—Dog heart-lung preparation. The effect on the heart rate of a preparation made from an untreated dog of 4 μg. of noradrenaline (A), and of 4 μg. of noradrenaline after the addition of 10 mg. of cocaine to the blood (B). Curve C shows the response of another preparation made from an untreated dog to 4 μg. of noradrenaline. Curve D shows the response in a preparation made from a dog previously treated with reserpine to 4 μg. of noradrenaline.

Effect of Cocaine.—When cocaine was added to the blood in the venous reservoir of the heart-lung preparation it modified the action of sympathomimetic amines in the same way as treatment with reserpine. The effect of noradrenaline and of adrenaline in doses of 4 μg. was increased and that of tyramine was diminished. An example of the effect of cocaine on the action of noradrenaline is shown in Fig. 6, in which curve **A** shows the effect before the addition of cocaine to the reservoir, and curve **B** shows the effect after the addition. The diminution of the action of tyramine is shown in Fig. 7. Curve **A** shows the effect of 1 mg. tyramine injected before the addition of cocaine, and curve **B** shows the effect after the addition.

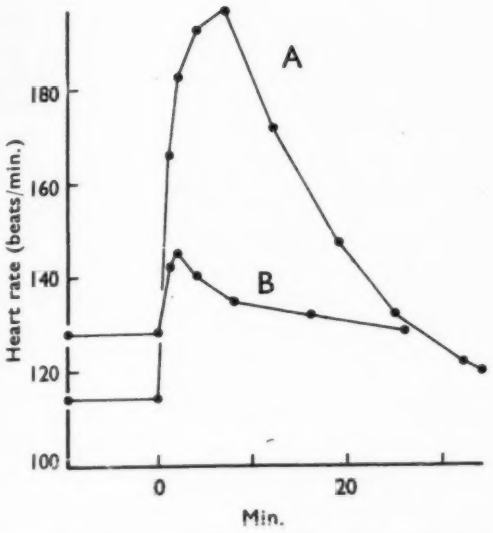

FIG. 7.—Dog heart-lung preparation made from an untreated dog. The response to 1 mg. of tyramine before A and after B the addition of 5 mg. of cocaine to the blood.

Effect of Hexamethonium.—After the injection of a large amount of hexamethonium, up to a total of 50 mg., the action of phenylethylamine, amphetamine and tyramine was similar to their action in other normal preparations. The action of noradrenaline appeared to be slightly increased.

Effect of Neosynephrine and 5-Hydroxytryptamine.—When neosynephrine was injected in amounts up to 0.2 mg. the increase in heart rate was no more than 5 beats/min. We also confirmed the finding of Paasonen and Krayer (1958) that the injection of 5-hydroxytryptamine in amounts up to 0.2 mg. did not increase the heart rate.

DISCUSSION

Bertler, Carlsson, and Rosengren (1956) showed that when rabbits were treated with reserpine, the catechol amines present in the heart disappeared, and Paasonen and Krayer (1958) have shown that this also occurs in the dog. They gave 0.5 mg./kg. of reserpine by intraperitoneal injection on two consecutive days and found that the noradrenaline was reduced to about 2% of the control value. The adrenaline content of normal dog hearts was very much lower (0.04 μg./g. as compared with 1.2 μg./g. for noradrenaline) and did not decline as a result of treatment with reserpine.

Our observations show that in the heart-lung preparation of the reserpine-treated dog, the group of sympathomimetic amines which is formed by phenylethylamine, phenylethanolamine and their derivatives (a group which includes tyramine and ephedrine) have lost their action. This action in the normal preparation seems therefore to be due to the release of noradrenaline from cardiac tissue. The action of the catechol amines, with the exception of that of dopamine, is, however, greater in the preparation from the reserpine-treated animal.

Certain differences were observed in the action of the amines on normal hearts. The action of phenylethylamine reached its peak within the first minute after injection and then declined, being finished in less than 10 min. The action of tyramine took nearly 10 min. to reach its peak and lasted up to 40 min., the time varying according to the dose. This may indicate that the —OH group in tyramine slows the rate at which it enters the cells. Both isomers of phenylethanolamine reached their maximum effect very quickly and acted longer than phenylethylamine. The action of the (+)-isomer was more prolonged than that of the (−)-isomer, as was observed on the blood pressure (Burn and Rand, 1958c). Amphetamine and ephedrine both exerted an effect so prolonged that it was impossible to determine the duration. Apart from the difference between the action of tyramine and that of phenylethylamine, the other differences in the duration of action appear to be explicable on the assumption that the duration depended on the rate of destruction by amine oxidase.

While the failure of these substances to act in the reserpine-treated preparation was clear enough, the sensitization of this preparation to noradrenaline was not so immediately obvious as when studies were made on the blood pressure. However, careful examination showed that the preparation which had been freed from noradrenaline by reserpine was more sensitive to noradrenaline. Hence the evidence from the heart supported the suggestion already made by Burn and Rand (1958c) that the store of noradrenaline in the blood vessel wall continuously discharges some noradrenaline, and that this noradrenaline occupies some of the receptors, creating some degree of tone. By so doing it reduces the effect of noradrenaline from outside. In the same way the store of noradrenaline in the heart would appear to discharge noradrenaline, increasing the rate of the pacemaker. In his observations on the effect of veratramine in abolishing the effect of adrenaline on the heart rate, Krayer (1950) observed that when full doses of veratramine were given the heart rate was sometimes much lower than it was initially, and pointed out that this might indicate the effect of

catechol amines released from within the heart in raising the pacemaker rate. Burn and Rand (1958a) found that atria from reserpine-treated rabbits beat at a mean rate of 112/min. in contrast to a rate of 146/min. for untreated controls.

It is of interest that the heart-lung preparation of the dog treated with reserpine resembles the preparation from an untreated dog after the addition of cocaine to the blood. After the addition of between 5 and 10 mg. cocaine, the effect of noradrenaline and of adrenaline is increased within a few minutes while the effect of tyramine and similar amines is greatly reduced. The effect of cocaine is not due to the dispersal of noradrenaline, at least not from the walls of the blood vessels. Burn and Rand (unpublished observations) injected large amounts of cocaine on two consecutive days into five rabbits, in doses of up to a total of 120 mg./rabbit. The amount of noradrenaline in the wall of the aorta remained normal. Burn and Rand (1958c) suggested that cocaine might stop the discharge of noradrenaline from the store in the organ. This would explain the inactivity of amines like tyramine which release noradrenaline, since cocaine would block the release. Rosenblueth and Schlossberg (1931) failed to observe that cocaine sensitizes the heart to the accelerating action of adrenaline; this may have been because the sensitization is less evident when the dose of adrenaline is large.

The addition of even so large an amount of hexamethonium as 50 mg. did not modify the action of amphetamine in causing a prolonged rise of heart rate. This result speaks against the conclusion of Reinert (1957) that amphetamine has a nicotine-like action.

We observed that the very small rise in rate which tyramine caused in the reserpine-treated heart became much greater after the slow intravenous infusion of noradrenaline. This result was comparable to the increase in the constrictor effect of tyramine in the perfused hindleg of the dog which followed a similar infusion of noradrenaline (Burn and Rand, 1958c). Both these observations indicate that the store of noradrenaline in the heart and in the vessel wall can be refilled from noradrenaline circulating in the blood. Burn (1932) observed the same effect in the blood vessels after an infusion of adrenaline. How far the refilling of the store by the amines circulating in the blood is a normal physiological phenomenon is not known. v. Euler (1956) failed to observe any increase in the catechol amine content of the heart after infusing noradrenaline into the cat at rates up to 8 μg./kg./min. for more than 30 min. Moreover, Goodall (1951) showed that the store in the heart is greatly depleted after removing the stellate ganglia: the depletion occurred after 2 weeks in animals in which the adrenal glands were present.

In conclusion, attention must be called to dopamine. Its action is diminished in the reserpine-treated heart-lung preparation, and the action is increased by an infusion of noradrenaline as is the action of tyramine. Hence, in the heart dopamine belongs to the class of drugs which release noradrenaline. In the vessels, however, dopamine, like noradrenaline, was found to act directly and to have a greater action in the reserpine-treated preparation than in the normal preparation.

We wish to express our indebtedness to the Wellcome Trust for providing the Cossor electrocardiograph (model 1314) with which the observations were made.

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EFFECT OF INHIBITORS OF CHOLINE ACETYLATION ON ACETYLCHOLINE OUTPUT AND MOTILITY IN RESPONSE TO ANTICHOLINESTERASES AND TO DISTENSION OF THE LUMEN OF ISOLATED GUINEA-PIG ILEUM

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Some pharmacological effects of acyl-aryl compounds, which inhibit the enzymatic acetylation of choline, have been studied on the isolated guinea-pig ileum. Diphenylylbutylacetic acid (M.G. 1763) greatly reduced the output of acetylcholine from intestine. The intestinal contractions produced by anticholinesterase drugs were inhibited, while the response to acetylcholine itself was little affected. The contraction of longitudinal muscle elicited by raising intra-luminal pressure was also decreased, while the emptying phase of the peristaltic reflex was scarcely affected. There was a correlation between inhibitory activity and chemical structure.

In previous work (Garattini, Morpurgo, and Passerini, 1958) some acyl-aryl compounds were shown to inhibit acetylcholine synthesis *in vitro* using choline acetylase extracted from rabbit brain, and evidence was advanced that this inhibition was effected by preventing acetylation of coenzyme A. In order to study the pharmacological actions of these compounds, experiments have been performed on the isolated intestine which is known to synthesize large amounts of acetylcholine (Feldberg and Lin, 1949).

The effect of these drugs has been studied both on the release of acetylcholine from isolated guinea-pig ileum in the presence of eserine, and also on the intestinal contractions produced by anticholinesterase drugs which have been attributed to the accumulation of acetylcholine in the wall of the intestine (Koelle, Koelle and Friedenwald, 1950; Shelley, 1955; Admiral, Myers and Van Houten, 1955). Other experiments were performed on the peristaltic reflex elicited by distension of the lumen in the isolated guinea-pig ileum since it has been shown that distension of the lumen provokes an increased output of acetylcholine (Chujyo, 1952) which is followed by a contraction of the longitudinal muscle.

METHODS

Assay of Acetylcholine Released from the Isolated Ileum of the Guinea-pig.—Guinea-pigs were killed by a blow on the neck and bled. The distal portion of ileum was rapidly removed and a segment about 15 cm. in length was washed, tied at both ends, and incubated in 20 ml. oxygenated, Mg-free Tyrode solution at 37°, and eserine sulphate was added (10 μg./ml.). Every 30 min. the bath fluid was exchanged and assayed for acetylcholine on the eserinized frog rectus muscle. At the end of the experiment, the intestine was taken out, blotted with filter paper, and weighed. The release of acetylcholine was expressed as μg./g. of tissue.

Responses of the Isolated Ileum of the Guinea-pig to Anticholinesterase Drugs.—Segments of guinea-pig ileum, about 3 cm. long, were suspended in a 20 ml. bath containing oxygenated Tyrode solution at 37°. Responses of the preparation to the drugs were recorded by a lever writing on a smoked paper.

After some preliminary experiments with eserine sulphate, neostigmine chloride (Prostigmin chloride) and pyridostigmine bromide (Mestinon, Roche) (Fromherz and Pellmont, 1953; Randall, Conroy, Ferruggia, Kappell, and Knoeppel, 1955), pyridostigmine was selected for use in these experiments because, in concentrations of 10^{-6} in the bath, it gave regular responses, and the intestine relaxed more rapidly after washing than with the other drugs. Anticholinesterases were given every 10 min., and test substances were added 1 min. before.

Peristaltic Reflex.—Segments of intestine about 7 cm. long were suspended in a 25 ml. bath containing oxgyenated Tyrode solution ($MgCl_2$ 0.01 g./l.), according to the method described by Trendelenburg (1917). The peristaltic reflex was elicited by distension of the intestinal lumen by a pressure of 3 cm. of Tyrode solution. The rise in intraluminal pressure was maintained for 1 min. and repeated every 5 min. Test compounds were added to the bath 1 min. before stimulation. Intestinal responses were recorded by two levers, one recording contractions of the longitudinal muscle, and the other the intestinal volume.

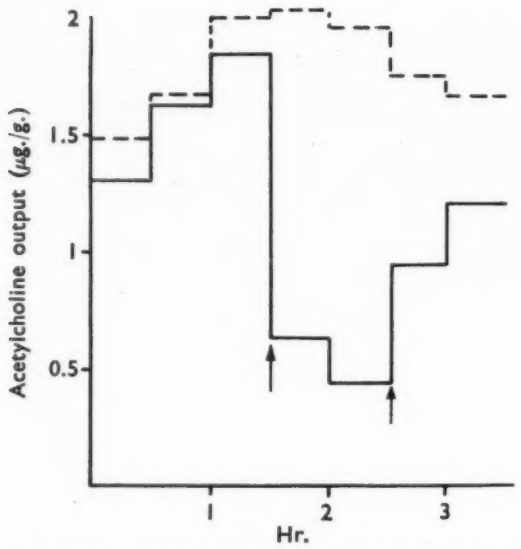

FIG. 1.—Output of acetylcholine from the isolated guinea-pig ileum. Ordinate: acetylcholine output in μg./g. Abscissa: time in hr. Assays of acetylcholine output were made every 30 min. Broken line, control acetylcholine output. Continuous line, acetylcholine output in experiments in which M.G. 1763 was added at the first arrow and the preparation was washed one hour later at the second arrow.

Drugs.—Many acyl-aryl compounds were assayed. This paper reports results obtained with some of the most active substances, namely, diphenylylacetic acid derivatives and diphenylheptylglycollic acid. They were used in the form of the sodium salts.

RESULTS

Action of Sodium Diphenylylbutylacetate (M.G. 1763) on the Output of Acetylcholine from the Isolated Guinea-pig Ileum.—When the acetylcholine release from the intestine in the control samples was high and constant, sodium diphenylylbutylacetate was added to the bath in a concentration of 20 μg./ml. 90 min. after it had been placed in the bath. The results are shown in Fig. 1. The broken line represents average amounts of acetylcholine released from the intestine in 8 control experiments. The continuous line represents the average of 4 experiments with M.G. 1763, added at the first arrow and washed out after 1 hr. Acetylcholine production was much reduced, but recovery was rapid when the drug was washed out.

Effect of Sodium Diphenylylbutylacetate on the Intestinal Responses to Different Anticholinesterase Drugs.—When an anticholinesterase was added to the bath, after a latency of a few seconds contraction of the intestine gradually developed which persisted until the preparation was washed. Larger doses of pyridostigmine were required than of eserine or neostigmine, and the latent period was also a little longer.

Fig. 2 shows the effect of M.G. 1763 (10 μg./ml.) on the intestinal response to eserine (0.5×10^{-6}), neostigmine (0.5×10^{-6}) and pyridostigmine (2×10^{-6}). M.G. 1763 greatly reduced the contractions caused by eserine and neostigmine and completely abolished that caused by pyridostigmine.

Effect of Various Inhibitors of Acetylcholine Synthesis on Intestinal Contractions Produced by Anticholinesterases.—Experiments were next performed to see whether all the compounds inhibiting acetylcholine synthesis *in vitro* were able to antagonize the response of the intestine to anticholinesterases, and whether there was a relationship between activity and chemical structure.

Fig. 3 shows the inhibition by sodium diphenylheptylglycollate (L.10) of the contractions of the isolated guinea-pig ileum induced by pyridostigmine. L.10, which strongly inhibited enzymatic acetylation *in vitro*, is thus very effective in this *in vivo* test.

Various diphenylylacetic acid derivatives were compared for their capacity to reduce the intestinal response to pyridostigmine. The results are summarized in Fig. 4, which shows that increasing length of the side chain can be correlated with increased inhibitory power.

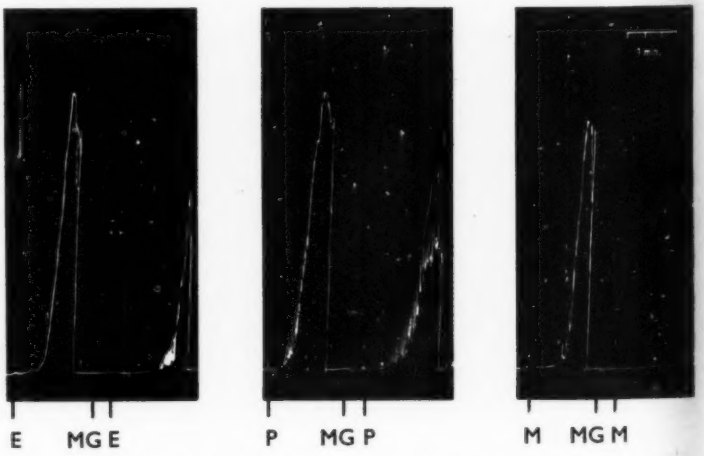

FIG. 2.—The isolated guinea-pig ileum. E, eserine 0.5×10^{-6}; P, neostigmine 0.5×10^{-6}; M, pyridostigmine 2×10^{-6}; MG, diphenylylbutylacetic acid (M.G. 1763) 10 μg./ml. which was added 1 min. before the second dose of each anticholinesterase.

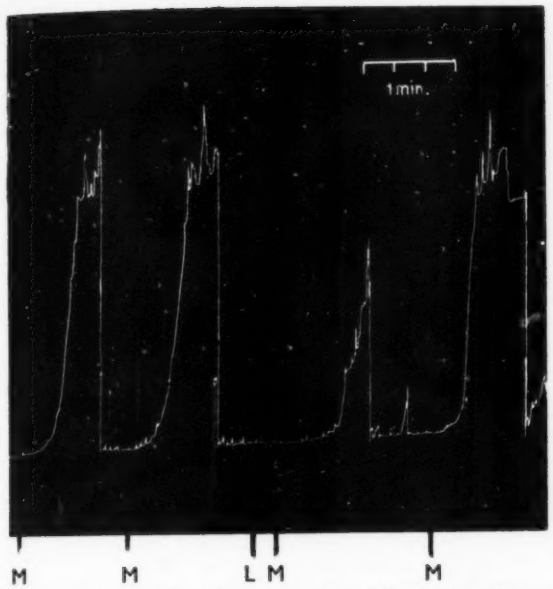

FIG. 3.—Isolated guinea-pig ileum. M, pyridostigmine 10^{-6}; L, diphenylheptylglycollic acid (L.10) 5 μg. ml. Pyridostigmine was added to the bath every 10 min. and allowed to act for 3 min. L.10 was added 1 min. before pyridostigmine.

Comparison of Sodium Diphenylylbutylacetate with Atropine on the Response of the Isolated Guinea-pig Ileum to Acetylcholine and Pyridostigmine.—In order to demonstrate that the compounds tested inhibited intestinal contractions by preventing acetylcholine synthesis and not by antagonizing acetylcholine, they were compared with atropine.

As shown in Fig. 5, M.G. 1763 in a concentration of 100 μg. in 20 ml. scarcely affected the contraction caused by 0.5 μg. of acetylcholine, but strongly inhibited that caused by 20 μg. of pyridostigmine. Atropine, in a dose of 0.05 μg., which reduced by about 50% height of the contraction caused by the same dose of acetylcholine, scarcely affected the response of the intestine to pyridostigmine.

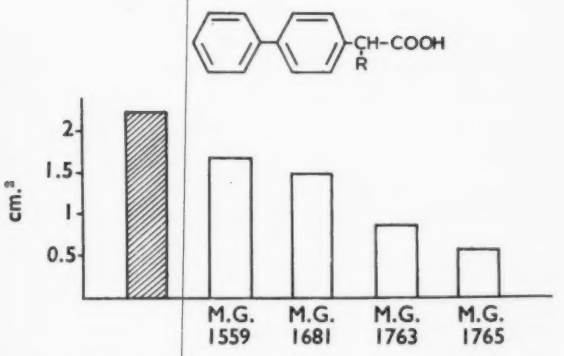

FIG. 4.—Effect of various diphenylylacetic acid derivatives (at a concentration of 10^{-5}) on the responses of isolated guinea-pig ileum to pyridostigmine (10^{-6}, applied for 2·5 min.). The values given are obtained by planimetry of contractions recorded on the tracing. The shaded area represents average value of controls. In the formula above the graph—R in M.G. 1559 is ethyl, in M.G. 1681 is propyl, in M.G. 1763 is butyl and in M.G. 1765 is pentyl.

Effect of Sodium Diphenylylbutylacetate (M.G. 1763) and Sodium Diphenylylethylacetate (M.G. 1559) on the Peristaltic Reflex.—M.G. 1763 inhibited the contraction of the longitudinal muscle responsible for the preparatory phase of peristaltic reflex, but the contractions of the circular muscle associated with the emptying phase were little affected. With increasing doses of

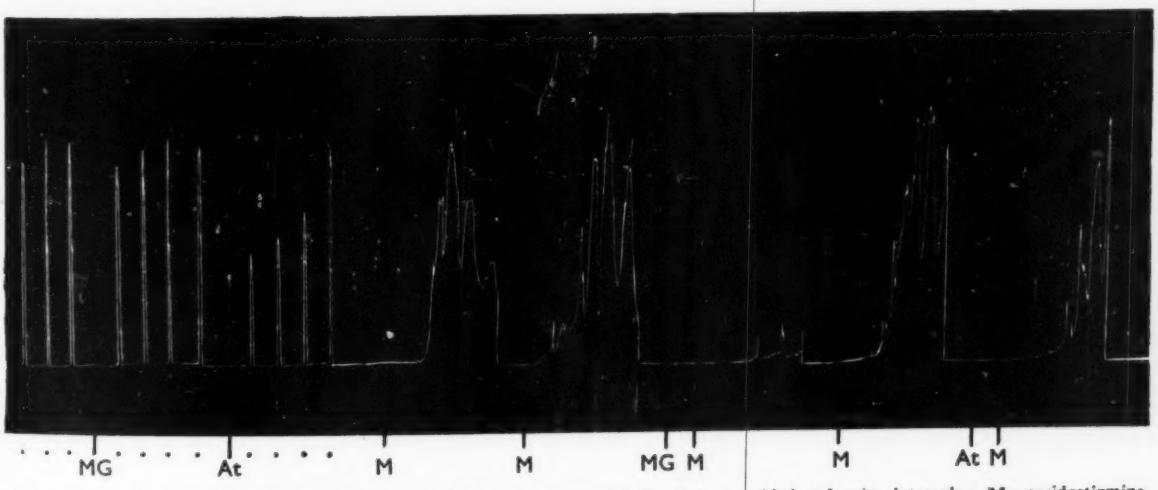

FIG. 5.—Isolated guinea-pig ileum in 20 ml. Tyrode solution. ·, = acetylcholine 0·5 μg., added at 3 min. intervals; M = pyridostigmine 20 μg. added at 10 min. intervals (contact time 5 min.); MG = diphenylylbutylacetic acid (M.G. 1763) 100 μg. and At = atropine 0·05 μg. Inhibitors were added 1 min. before stimulating drugs.

M.G. 1763 large inhibitions were obtained. The inhibition, which was reversible, could be repeated. M.G. 1559, a compound with a shorter side-chain, was again less effective than M.G. 1763.

Discussion

The results obtained in the present experiments are consistent with the *in vitro* findings, that aryl derivatives of acetic acid are able to inhibit the acetylation of choline.

The output of acetylcholine from the isolated ileum was much reduced by the presence in the bath fluid of diphenylylbutylacetic acid, one of the most powerful of these compounds. At a high concentration of M.G. 1763, the inhibition was complete, but it was always reversed by washing. A similar reduction in acetylcholine output was shown for morphine (Schaumann, 1957), but the inhibition was never complete even at high doses, and morphine was ineffective in inhibiting the synthesis of acetylcholine. The reduction in acetylcholine output produced by morphine was accounted for by a reduced acetylcholine release.

Our compounds inhibited the intestinal contractions produced by anticholinesterase drugs, and this inhibition does not seem to occur by an atropine-like mechanism, since the responses to acetylcholine were not or only slightly affected by atropine; neither was there any *in vitro* inhibition of anticholinesterase activity (Morpurgo, unpublished observation).

In the Trendelenburg preparation, our compounds were found to inhibit the longitudinal contraction of the ileum, while the peristalsis was scarcely affected. Explanation of this observation is difficult because the mechanisms involved in the different phases of the peristaltic reflex are not yet fully ascertained. The effects of different inhibitors have been examined (Feldberg and Lin, 1949; Kosterlitz and Robinson, 1957; Schaumann, 1955). Thus local anaesthetics and ganglion-blocking agents inhibit the circular contractions of the emptying phase, showing its nervous origin, while the contractions of the longitudinal muscle persist, probably because they are myogenic. Atropine and morphine affect both preparatory and emptying phases. As to the inhibition of longitudinal contractions exerted by our compounds, they may prevent the synthesis of acetylcholine by the intestinal muscle, since it has been shown that the acetylcholine in the intestinal wall is largely synthesized by muscle and not by nervous structures (Feldberg, 1949).

A relationship between the inhibitory activity on intestinal contractions and chemical structure was also shown by these as well as by the *in vitro* experiments. This supports the hypothesis that the phenomenon is due to the inhibition of acetylcholine synthesis.

Diphenylylacetic acid derivatives (M.G. compounds) were synthesized in Maggioni Laboratories, Milan (Cavallini, Massarani, Nardi, and Ambrosio, 1957), and diphenylheptylglycollic acid (compound L.10) was supplied by Carani in Lofarma Laboratories, Milan.

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THE DEPRESSOR ACTION OF DOPAMINE AND ADRENALINE

BY

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Dopamine is a pressor agent in the spinal cat, but causes a fall of blood pressure in the guinea-pig and rabbit under urethane anaesthesia. When guinea-pigs and rabbits are injected with reserpine, which depletes the vessel walls of noradrenaline, dopamine then becomes pressor. If an intravenous infusion of noradrenaline is given the depressor action returns. An intravenous infusion of vasopressin does not have this effect.

A strip of rabbit aorta is caused to contract by noradrenaline and by dopamine, but if dopamine is added at the height of a noradrenaline contraction it causes relaxation.

Adrenaline causes a fall of blood pressure in the cat under ether with vagi cut. However, in a reserpine-treated cat its action is pressor. The depressor action is restored during an infusion of noradrenaline. Noradrenaline has thus been shown to cause a vasomotor reversal of dopamine and of adrenaline.

Dopamine, or 3-hydroxytyramine, belongs to the group of sympathomimetic amines which contains adrenaline and noradrenaline, all the members of this group being derivatives of catechol. When injected intravenously into the spinal cat dopamine has a pressor action. It was therefore a surprise that Holtz and Credner (1942) found that dopamine caused a fall of blood pressure in the guinea-pig and in the rabbit. Interest in this observation has been renewed by the work of Hornykiewicz (1958), who confirmed the results of Holtz and Credner (1942), and showed that the depressor action of dopamine in the guinea-pig was increased by the previous injection of iproniazid, which inhibits amine oxidase.

We have recently found that sympathomimetic amines such as tyramine and ephedrine depend for their action on the noradrenaline present in the walls of the arteries, and that when the noradrenaline is dispersed from the walls by the injection of reserpine, tyramine and ephedrine no longer cause a rise of blood pressure (Burn and Rand, 1958a and b). For reasons which will be discussed later we decided to examine the action of dopamine in guinea-pigs and rabbits treated with reserpine, to see if its depressor action was modified.

METHOD

Guinea-pigs were anaesthetized by injecting urethane intraperitoneally in a dose of 1.5 g./kg. Rabbits were anaesthetized by injecting urethane intravenously; the dose was variable, enough being given to abolish all reflexes. Reserpine was given intraperitoneally after dissolving it in 20% ascorbic acid, the total dose being about 10 mg./kg. for a guinea-pig, 6 mg./kg. for a rabbit, and 5 mg./kg. for a cat: this dose was usually divided and given on two days. Rabbits were decapitated by Sherrington's method and spinal cats were prepared by Dale's method.

RESULTS

Reversal of Dopamine Action in the Guinea-pig.—When dopamine was injected into a normal guinea-pig under urethane anaesthesia, it caused a fall of blood pressure as shown in Fig. 1*a*. This fall was proportional to the dose as was found by Hornykiewicz (1958). When the injection was made into a guinea-pig which had received reserpine on the previous day, there was a rise of blood pressure (Fig. 1*b*). The rise occurred when 80 μg. was injected as shown, or when 20 μg. was injected, being proportional to the dose.

We have already stated that in the reserpine-treated cat tyramine has no pressor action; we observed that the pressor effect was restored when an intravenous infusion of noradrenaline was given. We therefore gave an intravenous infusion of noradrenaline to the reserpine-treated guinea-pig, and, as shown in Fig. 1*c*, after 10 μg. of noradrenaline had been infused the injection of 80 μg. dopamine caused a fall of blood pressure.

Reversal of Dopamine Action in the Rabbit.—When dopamine was injected into a normal rabbit

under urethane anaesthesia it caused a fall of blood pressure in amounts from 0.1 to 0.4 mg., but when larger amounts were given a pressor phase was superimposed on the fall. Results in one rabbit are shown in Fig. 2*a*; when 0.8 mg. dopamine was injected the pressor phase was becoming evident. When the same injections were made in a rabbit previously treated with reserpine, the effect in every case was pressor as shown in Fig. 2*b*. Thus treatment with reserpine reversed the depressor action of dopamine in the rabbit as well as in the guinea-pig.

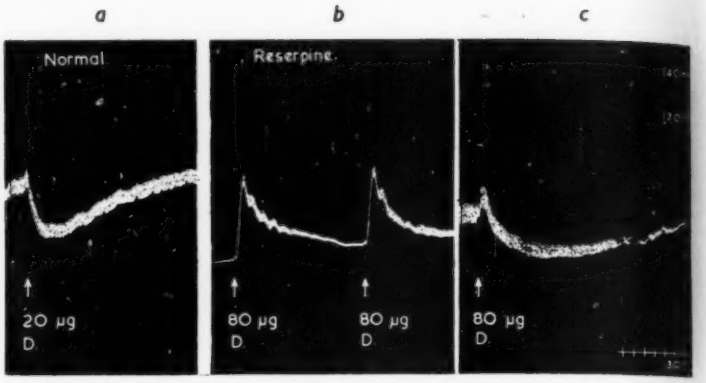


FIG. 1.—Guinea-pig blood pressure. (*a*) 20 µg. dopamine (D) given to normal guinea-pig i.v. (*b*) 80 µg. dopamine given to reserpine-treated guinea-pig. (*c*) 80 µg. dopamine injected after intravenous infusion of 10 µg. noradrenaline.

We took the opportunity to observe the action of isoprenaline on the blood pressure of the reserpine-treated rabbit. We saw that in one rabbit in which 0.1 mg. dopamine had a simple pressor action, the injection of 10 µg. isoprenaline caused a fall of blood pressure as in a normal animal. Similarly in the reserpine-treated guinea-pig isoprenaline in amounts of 0.2 and 2 µg. was depressor, though dopamine was pressor.

Conversion of Pressor to Depressor Effects.—The effects which we observed with dopamine were also observed with epinine, a substance in which the amine group of dopamine becomes methylamino. Epinine, while depressor in the normal rabbit in doses up to 0.2 mg., was pressor in the reserpine-treated rabbit as shown in Fig. 3*a*. When noradrenaline was given by slow intravenous infusion, the injection of 0.2 mg. epinine caused a fall of blood pressure (Fig. 3*b*). The infusion of noradrenaline was then stopped, and after a short time the pressor action of 0.2 mg. of epinine returned (Fig. 3*c*). An infusion of vasopressin (1 unit in 5 ml.) was then given, raising the blood pressure to the level during the infusion of noradrenaline. The injection of 0.2 mg. of epinine still caused a rise of pressure; its action was not reversed as during the infusion of noradrenaline. Similar observations were made with dopamine in reserpine-treated rabbits. Its pressor action persisted when it was injected during the infusion of vasopressin, but was reversed during an infusion of noradrenaline.

As already explained, when larger amounts of dopamine or of epinine were injected into normal

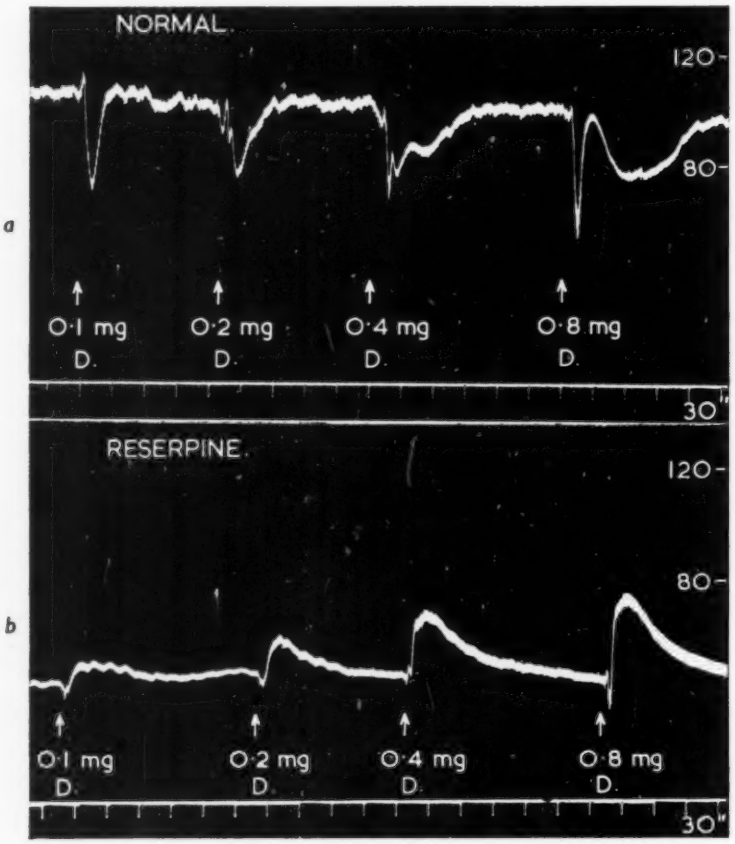


FIG. 2.—Rabbit blood pressure. (*a*) Injections of dopamine (D), 0·1 mg., 0·2 mg., 0·4 mg. and 0·8 mg. into normal rabbit. (*b*) A similar series of injections of dopamine into a rabbit treated with reserpine.

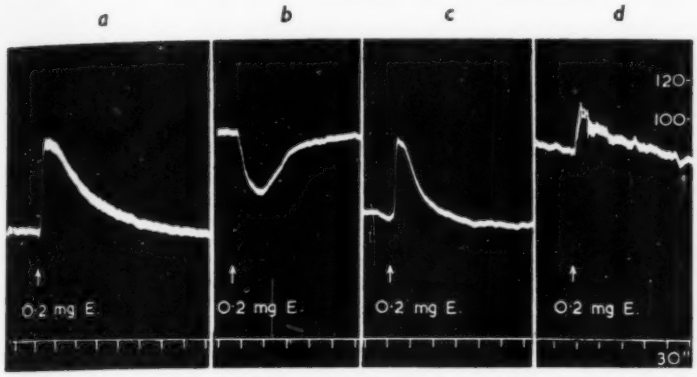


FIG. 3.—Blood pressure of rabbit treated with reserpine. (*a*) 0·2 mg. epinine (E). (*b*) 0·2 mg. epinine during infusion of noradrenaline. (*c*) 0·2 mg. epinine after infusion of noradrenaline. (*d*) 0·2 mg. epinine during infusion of vasopressin (1 unit in 5 ml.).

rabbits, a pressor response superimposed on the depressor action was recorded. This pressor response was caused to disappear by infusing noradrenaline. Thus in Fig. 4*a*, 1.6 mg. of dopamine caused a pressor effect in a normal rabbit. A noradrenaline infusion was then begun at a slow rate, the blood pressure not being very much raised. During the infusion the injection of 1.6 mg. of dopamine had the effect shown in Fig. 4*b*. When a total of 0.3 mg. of noradrenaline had been infused, the infusion was stopped. Over one hour later the injection of 1.6 mg. of dopamine had a simple depressor action. A similar result was obtained with epinine.

Dopamine in the Decapitate Rabbit.—The foregoing observations were made under urethane anaesthesia. We found that in a decapitate rabbit, although not previously treated with reserpine, the injection of dopamine had a pressor action as in the spinal cat. This pressor action, however, was abolished during the infusion of noradrenaline as shown in Fig. 5*a* and sometimes reversed to a depressor action. During the infusion of vasopressin, however, the pressor action of dopamine was sometimes greatly increased as shown in Fig. 5*b*.

Observations on Rabbit Aorta.—In order to reduce the number of factors concerned in changes in the blood pressure of the whole animal, we made observations on strips of rabbit aorta cut spirally and suspended in Locke solution in an 8 ml. bath. On each spiral we tested the effect of adding different amounts of noradrenaline to the bath and also of different amounts of dopamine. Both amines caused a contraction of the aorta. We also tested the effect of adding dopamine to the bath when noradrenaline had been added to the bath some minutes before and had produced a contraction which was sustained. In one experiment we observed that 16 μg. of noradrenaline caused a contraction of 67 mm. as recorded on the drum ; after the bath fluid was changed and the aorta was fully relaxed, the addition of 10 mg. of dopamine caused a contraction of 55 mm. When, however, 10 mg. of dopamine was added at the height of the noradrenaline contraction, it caused a partial relaxation of the aorta. We then studied the effect of adding 10 mg. dopamine to the bath after the addition of different amounts of noradrenaline. When 2 μg. of noradrenaline had caused its maximal effect, 10 mg. of dopamine caused a further contraction. When 5 μg. of noradrenaline had caused its maximal effect, 10 mg. dopamine had little effect. When 16 μg. of noradrenaline had caused its maximal effect, 10 mg. of dopamine caused relaxation as shown in Fig. 6, and a further 10 mg. of dopamine caused a greater relaxation. We observed these changes in each of six experiments.

Reversal in the Cat.—The ordinary view that dopamine is a pressor amine like adrenaline depends on its action in the spinal cat. Its action in this preparation is indeed like that of adrenaline since it is reversed, and becomes mainly depressor after the injection of ergotamine, as shown in Fig. 7*a*. Because dopamine is pressor in the spinal cat its depressor action in the guinea-pig and the rabbit might be regarded as a species

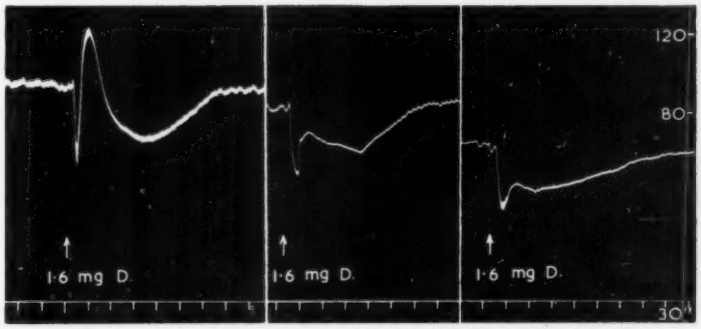


FIG. 4.—Blood pressure of normal rabbit. (*a*) 1·6 mg. dopamine (D). (*b*) 1·6 mg. dopamine during infusion of noradrenaline 5 μg./min. (*c*) 1·6 mg. dopamine given 77 min. after infusion of noradrenaline was stopped.

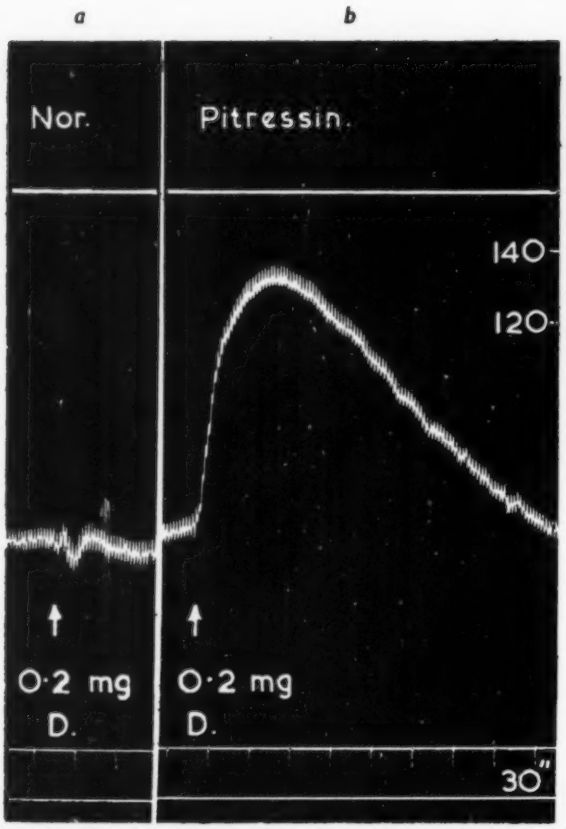


FIG. 5.—Normal decapitated rabbit in which 0·2 mg. dopamine (D) was pressor. (*a*) Absence of pressor effect of 0·2 mg. dopamine during noradrenaline infusion. (*b*) Greatly increased pressor effect of 0·2 mg. dopamine during infusion of vasopressin (Pitressin).

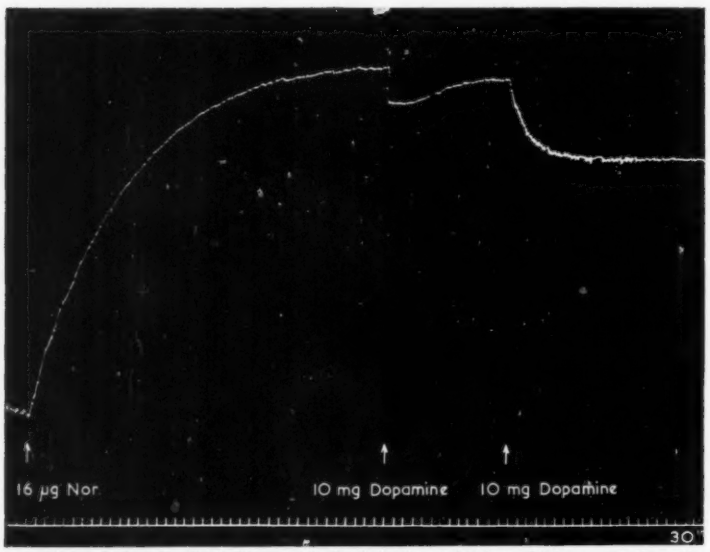


FIG. 6.—Spiral strip of rabbit aorta in 8 ml. bath. Addition of 16 µg. noradrenaline (Nor) caused a contraction, at the height of which 10 mg. dopamine caused a relaxation. The addition of another 10 mg. dopamine caused a further relaxation.

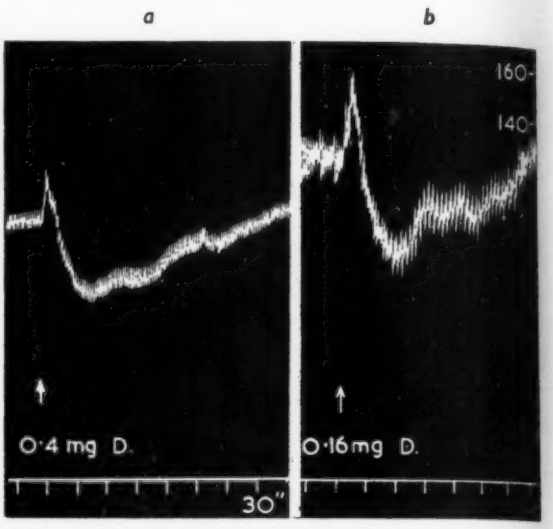


FIG. 7.—Cat blood pressure. (*a*) Spinal preparation after the injection of 2 mg. ergotamine; injection of 0·4 mg. dopamine (D) caused a fall of pressure. (*b*) Animal anaesthetized with urethane; injection of 0·16 mg. dopamine caused a fall in the blood pressure similar to that seen in (*a*).

difference. The difference seems to depend rather on the difference between the spinal preparation and the animal under urethane, for dopamine causes mainly a fall of pressure in the cat under urethane, and this fall resembles the fall of pressure in the spinal cat which has received ergotamine. Fig. 7*a* may be compared with Fig. 7*b*.

We have observed that the pressor action of dopamine in the spinal cat can be reversed to a depressor by a slow intravenous infusion of noradrenaline. In the experiment shown in Fig. 8, noradrenaline was infused for 38 min., the total infused being 3.23 mg. Before the infusion began 50 µg. dopamine had a pressor effect, and after the infusion 5 mg. dopamine caused a small but prolonged depressor effect. This was a large dose, but it is well known that the most marked depressor effect of adrenaline after ergotamine is seen when large doses such as 0.1 mg. are given, to which 5 mg. dopamine can be regarded as equivalent.

Depressor Action of Adrenaline.—Moore and Purinton (1900) described the depressor action of small doses of adrenaline in the anaesthetized cat. Dale and Richards (1918) showed that this fall was well seen in the cat anaesthetized with ether when the vagi were cut, and that it was attended

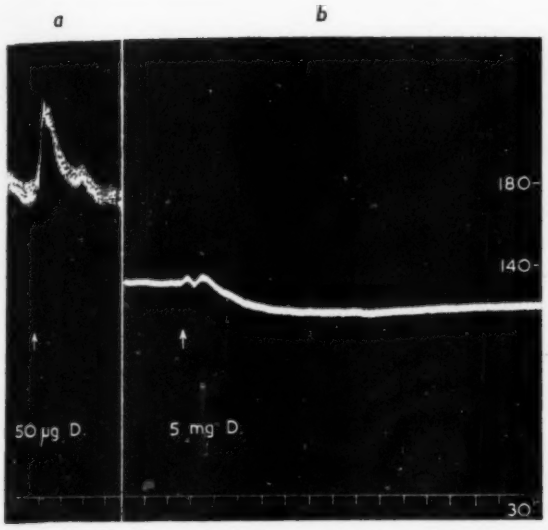


FIG. 8.—Blood pressure of spinal cat. (*a*) 50 µg. dopamine (D) caused a rise. An intravenous infusion of noradrenaline was then given during 35 min., the total being 3·23 mg. (*b*) After the end of the infusion, when the blood pressure was lower than in (*a*), the injection of 5 mg. dopamine caused a fall of blood pressure.

by dilatation of the hindleg in which the sciatic nerve had been cut some days previously. It seemed likely that the depressor action of dopamine would be evident under the same conditions. Fig. 9 shows a comparison of the effect of adrenaline with that of dopamine. The adrenaline was given by slow infusion from a burette during 2 min. Dopamine was given in the same way for a period of 80 sec., producing a much greater fall than was obtained with adrenaline.

In cats treated with reserpine, anaesthetized with ether and with the vagi cut, a slow intravenous infusion of adrenaline during 1 min. caused a rise of pressure (Fig. 10*a*). When a slow infusion of noradrenaline was given during 20 min. and during this period the infusion of adrenaline for 1 min. was repeated, there was a fall of pressure (Fig. 10*b*). After the infusion of noradrenaline was stopped and the blood pressure had fallen to a lower level than in Fig. 10*a*, the infusion of adrenaline now had only a slight pressor effect with a depressor component (Fig. 10*c*).

Observations were next made in cats in which the left sciatic nerve had been cut about 8 days previously. On the two days preceding the experiment the cats were injected with reserpine. They were then anaesthetized with ether, and the volume of the left hindleg was recorded by putting it in a plethysmograph, using a piston recorder. The vagi were cut. Adrenaline was then given intravenously, 5 µg. or 10 µg. being infused in 1 min. Whereas in a normal cat this would have caused a fall of blood pressure and dilatation of the hindleg, in reserpine-treated cats it caused a rise of blood pressure which was sometimes large and accompanied by constriction of the hindleg, but was usually small and without much change in leg volume as shown in Fig. 11 (*a*) and (*b*). A slow infusion of noradrenaline was then given during a period of 15 min. During this time when the blood pressure was not greatly raised, the slow infusion of 10 µg. and 5 µg. of adrenaline in 1 min. was repeated. It now caused a fall in blood pressure and dilatation of the leg as shown in Fig. 11 (*c*) and (*d*).

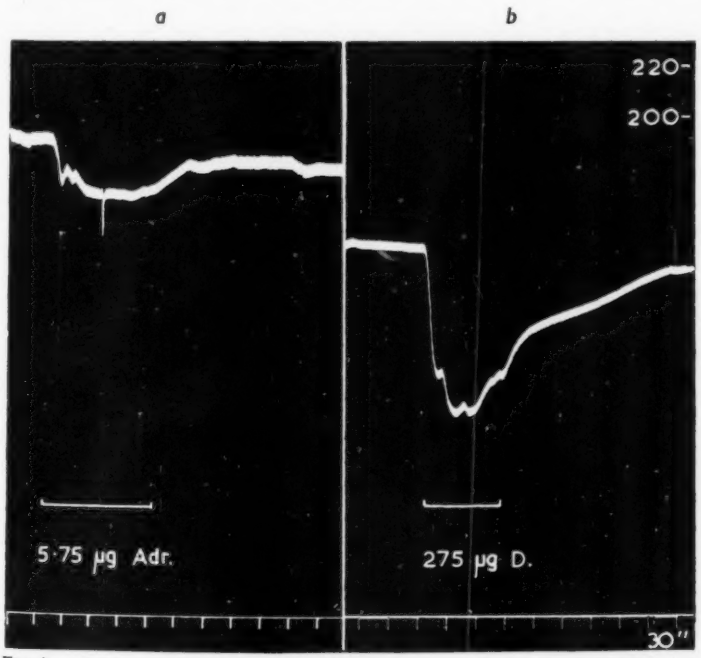


FIG. 9.—Blood pressure of cat under ether. Vagi cut. (*a*) Slow intravenous infusion of 5·75 µg. adrenaline (Adr) caused a fall of blood pressure. (*b*) Slow intravenous infusion of 275 µg. dopamine (D) caused a much larger fall of blood pressure.

Noradrenaline Stored in the Walls.—We extracted the aortae of eight cats to determine the amount of noradrenaline present. Four were cats under urethane and four were converted to spinal cats. We found no difference in the mean figure for noradrenaline in the two groups. The cats under urethane contained 0.295 µg./g. aorta, while the spinal cats contained 0.265 µg./g.

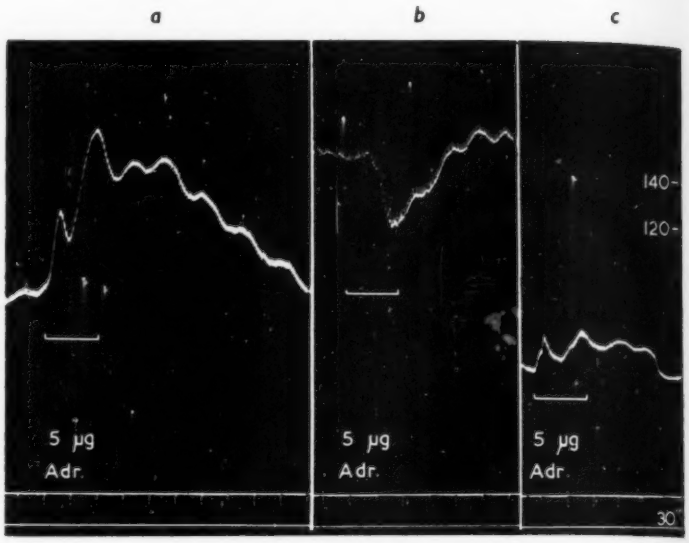


FIG. 10.—Blood pressure of cat treated with reserpine. Ether anaesthesia. Vagi cut. (*a*) Intravenous infusion of 5 µg. adrenaline (Adr) during 1 min. caused a rise of pressure. (*b*) During an infusion of noradrenaline which was given for 20 min., an infusion of 5 µg. adrenaline during 1 min. caused a fall of pressure. (*c*) After the infusion of noradrenaline was stopped, and the blood pressure had fallen below the level in (*a*), the infusion of 5 µg. adrenaline during 1 min. caused a much smaller pressor effect than in (*a*), and this effect had a depressor component.

Pressor Ratios of Noradrenaline, Adrenaline, and Dopamine.—We have shown (Burn and Rand, 1958b) that the pressor action of noradrenaline and other catechol amines is much increased in the spinal preparation of a cat which has been treated with reserpine, and that it is decreased after an intravenous infusion of noradrenaline. We compared the pressor activity of noradrenaline, of adrenaline, and of dopamine in two cats treated with reserpine; we found that in one the pressor action of adrenaline was 1/4 of that of noradrenaline, while that of dopamine was 1/100th of that of noradrenaline. In the other the action of adrenaline was 1/8 of that of noradrenaline, while that of dopamine was 1/150th of that of noradrenaline. These figures are different from those obtained using the spinal preparation of normal cats. Barger and Dale (1910) found the potency of adrenaline to be 2/3 or 4/5 of that of noradrenaline, while Gurd (1937) found the potency of dopamine to be about 1/30th of that of adrenaline.

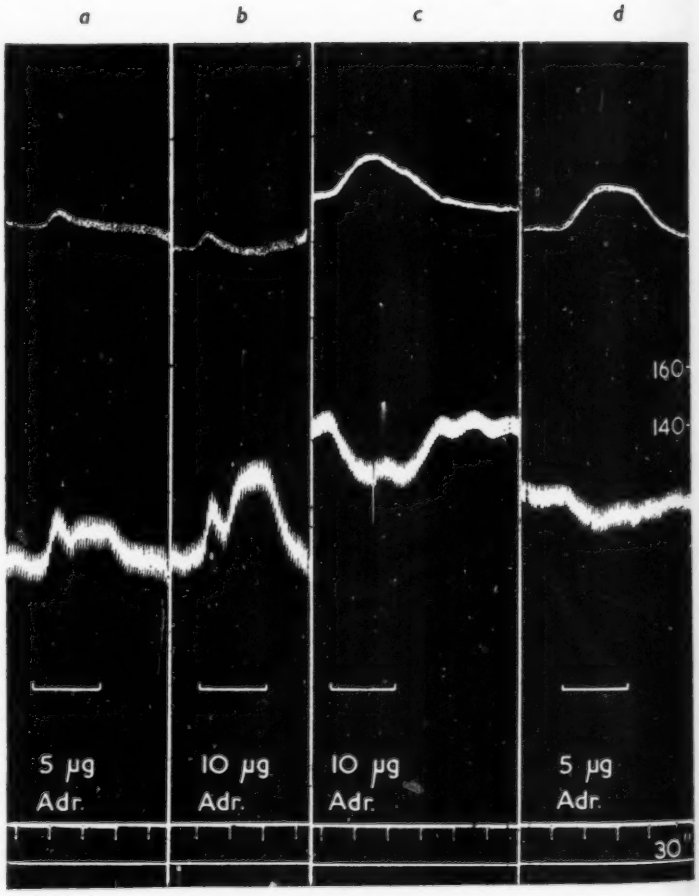
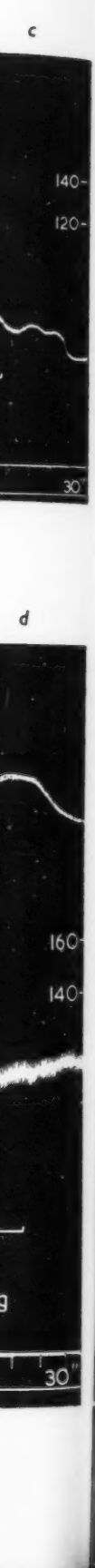


FIG. 11.—Cat with left sciatic nerve cut 8 days. Treated with reserpine. Ether anaesthesia, vagi cut. Upper record: volume of left hindleg. Lower record: blood pressure. (*a*) Infusion of 5 µg. adrenaline (Adr). (*b*) Infusion of 10 µg. adrenaline. A slow infusion of noradrenaline was then given. During this infusion (*c*) an infusion of 10 µg. adrenaline was also given, and (*d*) an infusion of 5 µg. adrenaline was also given. Note the pressor effect in (*a*) and in (*b*) accompanied by little change of leg volume. Note the depressor effect in (*c*) and (*d*) accompanied by dilatation of leg volume.



Discussion

When we first considered the depressor action of dopamine in the guinea-pig and in the rabbit, we had the idea that it might be related to the presence of noradrenaline in the vessel walls, both that which was liberated at sympathetic nerve endings and also that which was stored in the walls (Schmiterlöw, 1948). Dopamine is a much weaker constrictor agent than noradrenaline, its pressor action in the reserpine-treated spinal cat being only 1/100th or 1/150th of that of noradrenaline. We thought that an injection of dopamine into the normal guinea-pig or rabbit under urethane would set up competition between dopamine and noradrenaline for receptor sites. Dopamine would replace noradrenaline for some of these sites, but, being a much feebler constrictor agent, its occupation of these sites would result in a fall of vascular tone. We decided to test this view by treating animals with reserpine.

When an animal is treated with reserpine there is a diminution of sympathetic tone (Bein, 1955) and there is a disappearance of noradrenaline from the vessel walls (Burn and Rand, 1957). Consequently in the reserpine-treated guinea-pig and rabbit, dopamine when injected would find fewer receptor sites in the vessel wall occupied by noradrenaline, and would therefore produce a rise of blood pressure by occupying those which were free. This is what it was found to do.

The pressor action in the spinal cat of sympathomimetic amines like tyramine is absent if the cat is previously treated with reserpine, but it returns after an intravenous infusion of noradrenaline. Presumably this indicates that the intravenous infusion of noradrenaline replenishes stores of noradrenaline in the artery wall which are depleted by treatment with reserpine. If from the replenished store in the wall there is a small discharge of noradrenaline which occupies some of the receptor sites, then after an infusion of noradrenaline the injection of dopamine would again lead to competition between dopamine and noradrenaline for the receptor sites, and would produce a fall of blood pressure. It was in fact found that after an infusion of noradrenaline into the reserpine-treated guinea-pig and rabbit, dopamine once more had a depressor action.

That the effect of the infusion of noradrenaline was not due to a mere rise in vascular tone was evident from the observation that when an infusion of vasopressin was given which raised the blood pressure to the same extent, dopamine still exerted a pressor action. This observation was made in each of a series of experiments, and in one the pressor action of dopamine was greatly increased during infusion of vasopressin.

The depressor action of dopamine in the guinea-pig and rabbit under urethane has attracted attention because it stands in contrast to the pressor action seen in the spinal cat. The difference is, however, not due to the species, because dopamine is mainly depressor in the cat under urethane, and because it is pressor in the decapitate rabbit. The difference between the response of the cat under urethane and that of the spinal cat must be due to the noradrenaline which is liberated at sympathetic nerve endings to maintain tone. It was not due to a difference in the amount of noradrenaline stored in the artery wall, for no difference was found. However, when an infusion of noradrenaline was made into the spinal cat or into the decapitate rabbit, the pressor action of dopamine was first abolished and then reversed.

Observations on isolated strips of rabbit aorta have been in accord with this explanation of the pressor and depressor actions of dopamine. Both noradrenaline and dopamine were found to cause contractions of such strips; these contractions were similar in height when the dose of noradrenaline (in an 8 ml. bath) was 16 μg., and when the dose of dopamine was 10 mg. When, however, 10 mg. of dopamine was added to the bath at the height of the contraction caused by 16 μg. of noradrenaline, the aortic strip was partly relaxed.

These observations fit the ideas expressed by Ariëns (1954) that two substances may have similar "affinities" for receptors, but that their "intrinsic activities" when combined with the receptors may differ. Blocking agents are those which have an affinity for the receptors but which have no intrinsic activity. But another substance, which has some intrinsic activity when present alone, may nevertheless reduce the action of a substance having a higher intrinsic activity when it is present together with it. Stephenson (1956) has given a simple example of this in describing the action of butyltrimethylammonium and octyltrimethylammonium on the guinea-pig ileum. The former produced a large contraction in a given dose; the latter produced a small contraction in a much greater dose. However, when both substances were tested together in these same doses, the contraction was not much greater than that caused by the weaker substance acting alone. Stephenson called the weaker stimulant substance a "partial agonist." In his terms dopamine would be a partial agonist, that is a substance which though capable of combining with certain receptor

sites has only a feeble action when it does so. When such a partial agonist is in the presence of a more potent stimulant substance such as noradrenaline which combines with the same receptors, the partial agonist dopamine reduces the effect of the noradrenaline. This reduction can then appear as a fall of blood pressure; hence a fall is produced by a substance which in the absence of noradrenaline causes a rise of blood pressure. Thus the conception of the "partial agonist" provides an explanation for one form of vasomotor reversal.

The foregoing considerations seem to apply not only to the relation between noradrenaline and dopamine but also to the relation between noradrenaline and adrenaline. The relative pressor potency of these substances has been variously given; in the spinal cat Barger and Dale (1910) compared (±)-noradrenaline with (±)-adrenaline, and in different experiments found the ratio to be 1.5 : 1 and 1.25 : 1. During the present experiments we compared the pressor actions in the reserpine-treated cat, and found ratios of 8 : 1 and 4 : 1. Treatment with reserpine removes catechol amines, chiefly noradrenaline, from the artery wall, and the sensitivity of the wall to injected noradrenaline and adrenaline is then greater, particularly to noradrenaline.

In the cat under ether with the vagi cut, a slow infusion of a small amount of adrenaline, such as 5 μg., was found to cause a fall of blood pressure, and also a dilatation of the denervated hindleg (Dale and Richards, 1918). A similar slow infusion of noradrenaline caused a rise of blood pressure and constriction of the denervated hindleg (Burn and Hutcheon, 1949). If, however, the cat was treated with reserpine beforehand, then under the same conditions a slow infusion of 5 μg. of adrenaline caused a rise of blood pressure and constriction of the denervated hindleg. Again this effect was reversed by infusion of noradrenaline, adrenaline causing a fall of blood pressure and vasodilatation of the hindleg. It would therefore appear that the depressor action of small amounts of adrenaline, which was first observed by Moore and Purinton (1900) and which has been the subject of so much investigation, is to be explained in the same way as the depressor action of dopamine. The fall of blood pressure is due to the fact that when adrenaline is attached to receptor sites its efficacy as a constrictor is less than that of noradrenaline. Hence when the receptor sites are occupied by noradrenaline, the injection of adrenaline displaces noradrenaline from some receptors and a fall of blood pressure occurs as a result. However, as Fig. 9 shows, dopamine, being a much weaker constrictor agent than adrenaline, is much more effective in causing a fall.

Our evidence appears to explain one form of vasomotor reversal and so gives rise to the question whether the reversal of the action of adrenaline by ergotamine or by phenoxybenzamine can be similarly explained. We cannot make more than a passing comment on this. Ergotamine is a powerful vasoconstrictor substance (which retains its pressor action in the reserpine-treated spinal cat) and it is therefore possible that the ergotoxine-reversal of adrenaline described by Dale (1906) is similar to the noradrenaline reversal of dopamine. Phenoxybenzamine, however, is not a powerful constrictor substance itself, and it is not possible to suggest that in its presence adrenaline diminishes vascular tone because it is a weaker constrictor substance.

We have found that isoprenaline causes a fall of blood pressure in the reserpine-treated cat when dopamine causes a rise. Thus the effect of isoprenaline must be a direct effect, and implies that there are vasodilator receptors as Dale (1906) originally supposed. His hypothesis that ergotamine blocked the vasoconstrictor receptors, leaving the vasodilator receptors free, would therefore still apply to a substance like dibenzyline and be consistent with all the observations.

This work was done during the tenure by one of us (M. J. R.) of a Fellowship from the Life Insurance Medical Research Fund of Australia and New Zealand. We wish to thank Miss Roneen Hobbs for making the observations on the aorta strips.

Addendum

Sir Henry Dale pointed out to us that in the rabbit adrenaline is a pressor substance under all circumstances. We therefore tested the effect of adrenaline in the rabbit under ether before and during an infusion of noradrenaline. We found that it was pressor before the infusion, but had no effect in any dose during the infusion. We next compared the pressor action of adrenaline with that of noradrenaline in rabbits previously treated with reserpine. We found that adrenaline was equal in pressor action to noradrenaline, whereas in the reserpine-treated cat adrenaline had only one-sixth the pressor action. The relation in the rabbit seems to us to explain why adrenaline is not depressor in that animal.

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THE PERIPHERAL ACTION OF HEXAMETHONIUM AND OF PENTOLINIUM

BY

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The influence of hexamethonium and pentolinium on the responses of certain peripheral effector cells to adrenaline, noradrenaline or postganglionic stimulation was studied in the cat. The actions of adrenaline and noradrenaline on the blood vessels of a limb and of adrenaline and postganglionic stimulation on the nictitating membrane were increased after the administration of hexamethonium and pentolinium. This effect was considered to be due to sensitization of the peripheral effector cells. The possible significance of these findings is discussed.

Hexamethonium and pentolinium are quaternary ammonium compounds which block transmission in autonomic ganglia by competition with acetylcholine. From animal experiments it is known that, under standard conditions, these substances are extremely consistent in their behaviour and that the ganglionic synapse, no matter which ganglionic pathway is tested, shows an increasing sensitivity to subsequent doses. Repeated doses of hexamethonium and pentolinium, however, produce decreasing responses from the blood pressure (Zaimis, 1956). The same discrepancy between blood pressure and ganglionic responses is seen in man during the intravenous administration of these drugs for the reduction of blood pressure in operations; the first dose usually produces a good fall of blood pressure, but the pressure returns quite rapidly to its initial level and any subsequent doses are practically without effect (Enderby, personal communication). Also it has been the experience of clinicians that a daily increase in the dose of hexamethonium or pentolinium is necessary for the first few weeks if the initial rate of reduction in blood pressure is to be maintained. This is usually described as the development of "tolerance." In addition, there is the much discussed potentiation of adrenaline and noradrenaline which always occurs in the presence of hexamethonium or pentolinium, and which cannot be attributed only to the abolition of the normal compensatory mechanisms as it is found both after vagotomy and after section of the spinal cord at a high level (Bartorelli, Carpi and Cavalca, 1954; Salerno, 1955; Maengwyn-Davies, Walz and Koppanyi, 1958). All these factors taken together suggested the possibility of a peripheral action of hexamethonium and pentolinium in the form of a sensitization of the effector cells to adrenaline and noradrenaline (Zaimis, 1955). This hypothesis has been tested in the experiments reported in the present paper. Some of these results have already been reported briefly (Zaimis, 1956).

METHODS

Cats were anaesthetized with a mixture of chloralose (80 mg./kg.) and pentobarbitone (6 mg./kg.) injected into the cephalic vein of the fore limb or the saphenous vein of the hind limb. The addition of pentobarbitone prevented the initial stage of excitement which normally follows the intravenous injection of chloralose alone.

Recording of the Venous Outflow from the Hind Limb.—An incision was made in the skin of the medial surface of the thigh to expose the femoral artery and vein, and all branches of both femoral artery and vein down to the popliteal space, including the saphenous vein, were ligated and cut, with the exception of the artery chosen for the intra-arterial administration of the drugs. In order to maintain a regular blood-flow through the limb, the muscles were stimulated indirectly by the application of electrical shocks to the sciatic nerve at a frequency of 6 stimuli/min. Heparin (1,000 units/kg.) was administered intravenously 10 min. before any vessels were opened. The method described by Hilton (1952, 1953) was used for recording the venous outflow. In the tracings the height of the record measures the time interval between successive drops.

The drugs were injected either *close-arterially* by means of a cannula made from a No. 18 hypo-

dermic needle tied into the cut central end of a branch of the femoral artery, usually the small artery supplying the gracilis muscle; or *distant-arterially* into the external iliac artery of the non-operated leg, the cannula in which pointed towards the bifurcation of the aorta.

Contractions of the Nictitating Membrane.—These were elicited either by rectangular pulse stimuli of 0.3 msec. duration and applied at 10/sec. to the pre- or post-ganglionic cervical sympathetic nerve trunk; or by intra-arterial injections of adrenaline into the cut central end of the lingual artery.

Drugs.—(−)-Adrenaline tartrate, (−)-noradrenaline bitartrate, hexamethonium iodide and pentolinium bitartrate were used throughout these experiments and are referred to as adrenaline, noradrenaline, hexamethonium and pentolinium respectively.

RESULTS

Blood-flow.—In the first group of experiments, 0.1 μg. of noradrenaline was injected close-arterially at intervals of 10 to 15 min. This dose produced a local vasoconstriction with no change of the general blood pressure. After the intravenous administration of hexamethonium, or pentolinium, the vasoconstrictor effect of noradrenaline became more pronounced and more prolonged. Fig. 1 illustrates the results of two such experiments. In Fig. 1*a* 1 mg. of hexamethonium and in Fig. 1*b* 1 mg. of pentolinium was administered intravenously. This potentiating effect lasted for 10 to 15 min. with hexamethonium and somewhat longer with pentolinium.

In a second series of experiments noradrenaline was administered intravenously and hexamethonium or pentolinium distant-arterially. Fig. 2 illustrates such an experiment in which 1 μg. of noradrenaline administered intravenously produced a rise in blood pressure and a diphasic effect on the blood-flow through the limb, an increase to begin with because of the rise in the general blood pressure, followed by a decrease due to the direct vasoconstrictor effect of noradrenaline on the limb vessels. After the distant-arterial administration of 200 μg. of hexamethonium both the local vasoconstriction and

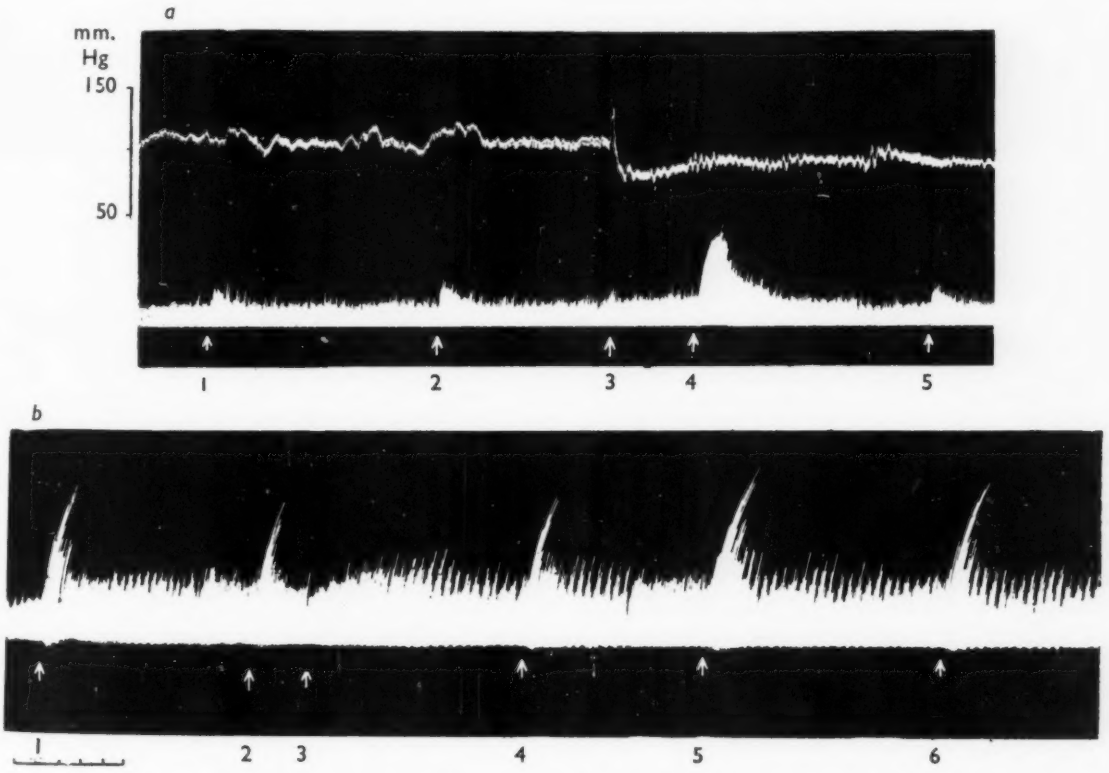

FIG. 1.—(*a*) Cat: 2 kg. Blood pressure upper trace. Venous outflow from hind limb lower trace. At 1, 2, 4 and 5, close-arterial injections of 0·1 μg. of noradrenaline. At 3, intravenous injection of 1 mg. of hexamethonium. (*b*) Cat: 3·2 kg. Venous outflow from hind limb. At 1, 2, 4, 5 and 6, close-arterial injections of 0·1 μg. of noradrenaline. At 3, intravenous injection of 1 mg. of pentolinium. Time, min.

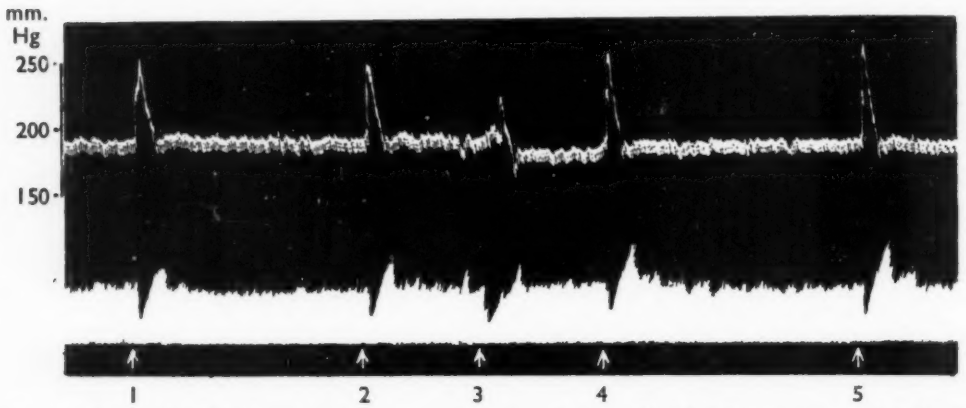

FIG. 2.—Cat: 2·6 kg. Blood pressure upper trace. Venous outflow from hind limb lower trace. At 1, 2, 4 and 5, intravenous injections of 1 μg. of noradrenaline. At 3, distant-arterial injection of 200 μg. of hexamethonium.

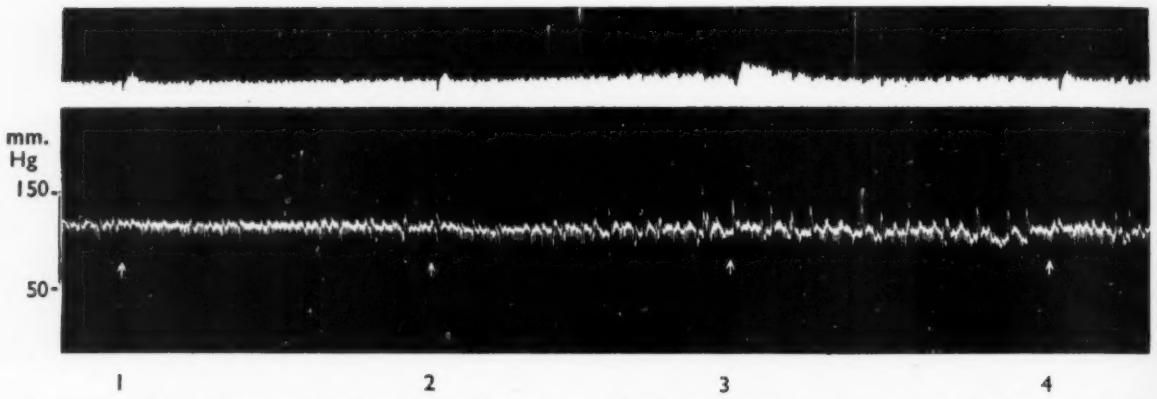

FIG. 3.—Cat: 3·6 kg. Venous outflow from hind limb upper trace. Blood pressure lower trace. At 1, 3 and 4, distant-arterial injections of 1 μg. of noradrenaline. At 2, distant-arterial injection of 1 μg. of noradrenaline together with 100 μg. hexamethonium.

blood pressure responses produced by noradrenaline were increased. In a third series of experiments both drugs were administered distant-arterially and similar results were obtained. After 100 μg. of hexamethonium, noradrenaline produced a more pronounced vasoconstriction (Fig. 3).

In a few experiments in which adrenaline was used, both hexamethonium and pentolinium increased its vasoconstrictor action.

Nictitating Membrane.—In the first group of experiments, a series of submaximal contractions of the nictitating membrane were elicited at regular time intervals by the electrical stimulation of the postganglionic cervical sympathetic trunk. After a number of control contractions hexamethonium or pentolinium was administered intravenously. The contractions increased after the administration of either drug. The onset of the effect was usually rapid, attained its maximum in about 3 to 6 min. and lasted for

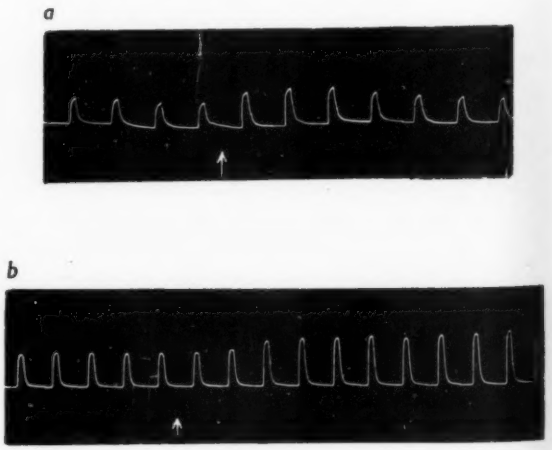

FIG. 4.—(*a*) Cat: 2·2 kg. Contractions of nictitating membrane elicited every 3 min. by submaximal postganglionic stimulation at 10 shocks/sec. Period of stimulation 30 sec. At arrow, intravenous injection of 2 mg. of hexamethonium. (*b*) Cat: 2·8 kg. Similar experiment. At arrow, intravenous injection of 2 mg. pentolinium.

10 to 15 min. for hexamethonium and somewhat longer for pentolinium. Fig. 4 illustrates two such experiments in which the potentiating effect was obtained with doses of hexamethonium or pentolinium which produced 50% or less ganglionic blockade. Larger doses did not alter the magnitude of the potentiating effect.

In a second series of experiments, contractions of the nictitating membrane were elicited by the intra-arterial administration of adrenaline in doses which produced submaximal responses. The intravenous administration of either hexamethonium or pentolinium was followed by an increased response (Fig. 5). This potentiation was produced by doses of hexamethonium and pentolinium comparable to those necessary to affect contractions elicited by postganglionic stimulation. The time course of the effect was also similar.

Discussion

Hexamethonium and pentolinium are powerful ganglionic blocking substances, and in order to detect peripheral action only tests which avoid ganglionic interference can be used. Such conditions are offered by the response of the blood vessels of a limb to adrenaline and noradrenaline, or the response of the nictitating membrane to postganglionic stimulation or to injected adrenaline.

The results reported above demonstrate that hexamethonium and pentolinium sensitized the

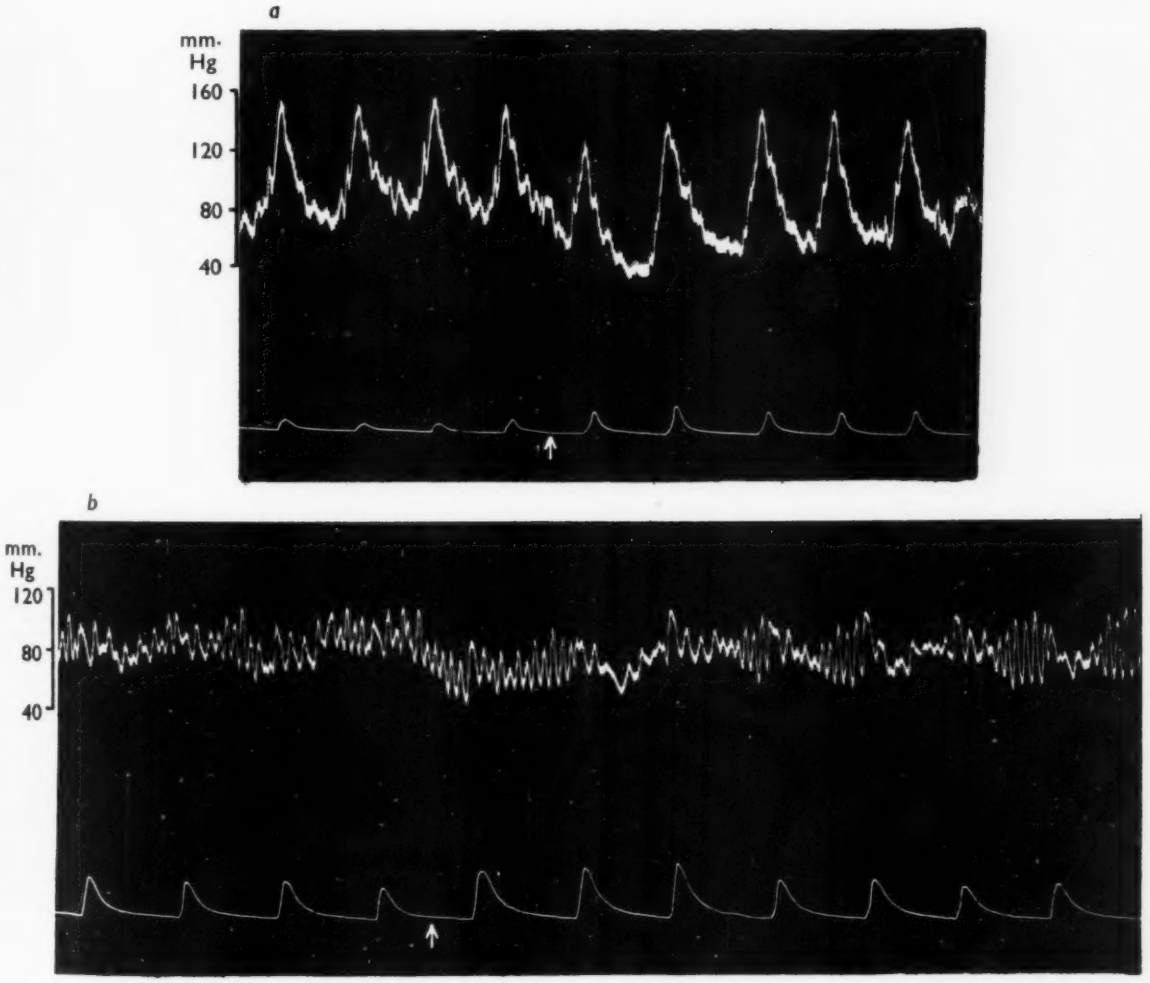

Fig. 5.—(*a*) Cat : 3·4 kg. Blood pressure, upper trace. Contractions of nictitating membrane elicited every 5 min. by intra-arterial injection of 2 μg. of adrenaline, lower trace. At arrow, intravenous injection of 2 mg. of hexamethonium. (*b*) Cat: 2·4 kg. Similar experiment but with intra-arterial injection of 1 μg. of adrenaline. At arrow, intravenous injection of 580 μg. of pentolinium.

effector cells to adrenaline, noradrenaline, and to sympathetic stimulation. This sensitization, although usually small, could always be detected, and it was independent of the route of administration of either the sympathomimetic amines or the ganglionic blocking drugs. The effect appeared with doses of hexamethonium or pentolinium smaller than those producing a complete ganglionic blockade. With repeated or larger doses the potentiating effect remained the same.

Evidence that quaternary ammonium compounds with powerful ganglionic blocking activity can increase the response of effector cells to chemical and mechanical stimuli can be found in several reports. Very often, however, such evidence has been misinterpreted or has passed unnoticed.

Shimamoto, Kanauchi, and Uchizumi (1955), in experiments on the nictitating membrane of the cat, found that both tetraethylammonium and hexamethonium potentiate the action of adrenaline, noradrenaline, and postganglionic sympathetic stimulation. This effect was present in normal as well as in denervated preparations and also after adrenalectomy. The same investigators reported that, under the influence of the two quaternary ammonium compounds, the relaxation of the isolated uterus of the cat and of the isolated sphincter pupillae muscle of cats and rabbits produced by adrenaline and noradrenaline was inhibited or even reversed. Discussing their results, Shimamoto *et al.* (1955) reached a conclusion which is rather difficult to understand. According to them, all these effects were produced because tetraethylammonium and hexamethonium "abolish the inhibitory action of adrenaline, noradrenaline, and postganglionic sympathetic stimulation." Perry and Wilson (1956), when studying the relative effects of ganglion blocking drugs on the sympathetic and parasympathetic supply to the heart in cats, found that pentamethonium was more active on parasympathetic ganglia and that the rapid onset of parasympathetic block which the drug produced was accompanied by a potentiation of the effect of sympathetic stimulation. They made the further observation that the potentiation of the sympathetic response was also present when the sympathetic stimulation was postsynaptic, indicating that this effect was a peripheral and not a ganglionic one. Bülbring (1955), using the isolated taenia coli of the guinea-pig, demonstrated that hexamethonium had a stimulant action and sensitized the muscle to different forms of stimulation.

Zauder (1954) found that hexamethonium potentiated the responses of the isolated intestine of the cat to both acetylcholine and histamine, an action which he considered was independent of innervation and probably due to "an increase in the excitability of the muscle in a manner as yet unexplained." Meier, Tripod, and Wirz (1957), using the perfused rabbit hind limb preparation, found that hexamethonium markedly increased the vasoconstrictor action of 5-hydroxytryptamine.

Thus it appears that hexamethonium and pentolinium can potentiate smooth muscle responses to different stimuli. It is difficult to say how this sensitization is brought about. Possibly these quaternary ammonium compounds produce permeability changes which in turn alter the behaviour of excitable cells in response to physiological and pharmacological stimuli.

From the results reported in this paper and from the evidence produced by other investigators, the conclusion may be drawn that subsequent doses of hexamethonium or pentolinium produce decreasing blood pressure effects, not because the magnitude of the ganglionic blockade is diminished, but because this is partially masked by the peripheral sensitization of the blood vessels to adrenaline and noradrenaline. Considering the extent of the vascular tree it is obvious that even a small increase in the response of the blood vessels to adrenaline and noradrenaline will produce pronounced haemodynamic consequences (Folkow, 1956). Sensitization of the blood vessels may well account for the increase in the responses produced by adrenaline and noradrenaline in the presence of hexamethonium or pentolinium, and for the development of "tolerance" which appears during the treatment of hypertensive patients.

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EFFECTS OF DRUGS WHICH DEPRESS THE PERIPHERAL NERVOUS SYSTEM ON THE RETICULAR ACTIVATING SYSTEM OF THE CAT

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Some drugs with depressant properties on the peripheral nervous system have been examined for depressant effects on the reticular activating system of the cat. Large doses of nicotine, or of the anti-nicotinic agents dihydro-β-erythroidine and mecamylamine, failed to depress the reticular activating system; non-quaternary drugs with anti-muscarinic properties, such as atropine and hyoscine, depressed it readily. Hyoscine was the most potent depressant tested and its effects could be antagonized by physostigmine. In contradistinction, depression of the reticular activating system with pentobarbitone was not antagonized by physostigmine. Lignocaine was a weak depressant of the reticular activating system, and the possibility that this might be due to a central anti-muscarinic action is discussed. Adrenergic blocking drugs, such as dihydroergotamine, phenoxybenzamine or choline 2 : 6-xylyl ether, did not appear to depress the reticular activating system: the significance of this is discussed. It was concluded that the hypothetical cholinergic transmitter, acting somewhere within the reticular activating system, displayed actions analogous to the muscarinic, and not to the nicotinic, actions of acetylcholine.

The discovery by Moruzzi and Magoun (1949) that electrical stimulation of the brain stem reticular formation is followed by behavioural and electroencephalographic signs of arousal in animals was an outstandingly important contribution to neurological science. The recognition of such a reticular activating system has provided neurophysiologists, and in particular Magoun and his associates, with a means of disentangling some of the complex reactions of the brain to the influx of sensory information ; it has stimulated neuroanatomists in their search for recognizable functional pathways inter-connecting cortex and midbrain ; and it has given neuropharmacologists a valuable tool for investigating the effects of drugs upon the elusive central synapse.

The demonstration that the reticular formation is particularly susceptible to the depressant effects of hypnotic and anaesthetic agents (French, Verzeano, and Magoun, 1953a ; Arduini and Arduini, 1954) has been followed by several useful analyses of the effects of various drugs on the reticular arousal system (for example, Domino, 1955 ; King, 1956 ; Gangloff and Monnier, 1957 ; Bradley and Key, 1958 ; Kuehn and Schallek, 1958). Other workers, notably Bradley and Elkes (1957), have studied the effects of drugs on the state of arousal of unrestrained, unanaesthetized animals, and have provided further indirect evidence of possible drug influences on the reticular system.

Evidence to date suggests that cholinergic neurones play an important rôle in the reticular activating system (Rinaldi and Himwich, 1955a, 1955b) and are hence to be considered along with other factors determining changes in the electrical pattern recorded from the cortex itself. There is no doubt that an adrenaline-sensitive component also exists within the reticular activating system (Bonvallet, Dell, and Hiebel, 1954 ; Rothballer, 1956).

In the present work some drugs which are known to depress the peripheral nervous system have been examined for possible depressant actions on the reticular activating system of the cat.

METHODS

Medium-sized cats were anaesthetized with ether and a tracheotomy tube inserted. A polythene tube for the injection of drugs was secured in the right femoral vein. Blood pressure was monitored

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routinely by a modified anaeroid gauge, carefully calibrated, which was connected to a polythene tube in the right femoral artery.

The skull was partly exposed and cortical potentials recorded on a Grass Model IV-A electroencephalograph; gramophone-needle electrodes (Hoagland, 1940) were inserted through the posterior walls of the frontal sinuses and dura (in order to make contact with the sensorimotor areas), and through burr holes drilled almost down to the dura over the marginal gyri. Electrocardiograms were recorded from needle electrodes placed in the forelimbs.

The head was fixed in a Lab-Tronics Model 4 stereotaxic instrument and a concentric stimulating electrode, constructed according to Jasper and Droogleever-Fortuyn (1947), was introduced through a hole in the skull and dura until its tip was positioned at Horsley-Clarke co-ordinates F+2, L 3, H−1. These were chosen after reference to the stereotaxic atlas of Jasper and Ajmone-Marsan (1954). The electrode tip was never moved outside a radius of 1 mm. from the selected point. In view of the consistently low stimulus threshold-voltage for electroencephalographic activation required at this point, and the latitude afforded by the relatively large area of reticular substance at this frontal plane, the placement of the electrode was not confirmed histologically as a routine.

At the end of the surgical procedures the external auditory meati and all wounds were infiltrated with 1% procaine solution; ether anaesthesia was discontinued, respiration supported artificially, and the preparation immobilized with serial injections of tubocurarine. Body temperature was maintained by infra-red radiation. The room was darkened and kept as quiet as possible in order to encourage drowsy or sleep-like electroencephalographic patterns.

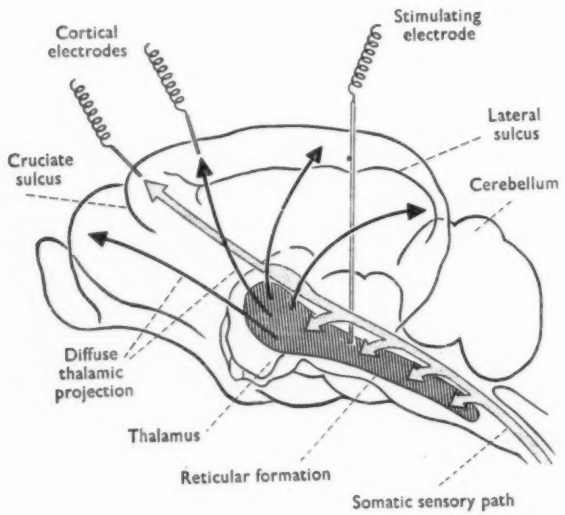

FIG. 1.—Diagrammatic representation of lemniscal and extralemniscal afferent systems radiating to cat cerebral cortex; approximate relation of stimulating and recording electrodes to these structures is shown.

After 2 hr., when the effects of ether had worn off, the treatment with local anaesthetic was repeated. The reticular formation was stimulated (not more than once every 3 min.) for 3 sec. periods with supramaximal rectangular pulses (6 V., 0.1 msec. duration, 250 shocks/sec.) through a Grass stimulator and isolation unit. One channel of the electroencephalograph was utilized for stimulus marking.

Observations on the arousal responses to sensory stimulation were made from time to time by stroking the back of the animal firmly with a glass rod.

Fig. 1 shows, in a diagrammatic form, the brain stem reticular formation, the diffuse projection from thalamus to cortex, the somatic sensory path and the sensory collateral connexions (Starzl, Taylor, and Magoun, 1951; French, Verzeano, and Magoun, 1953b) leading into the reticular formation. The approximate relation of stimulating and recording electrodes to these structures is also shown.

RESULTS

Electroencephalographic Response to Reticular Stimulation

Typical electroencephalographic activation changes are shown in Fig. 2, firstly in response to a 3 sec. period of electrical stimulation of the reticular formation, and secondly in response to touch.

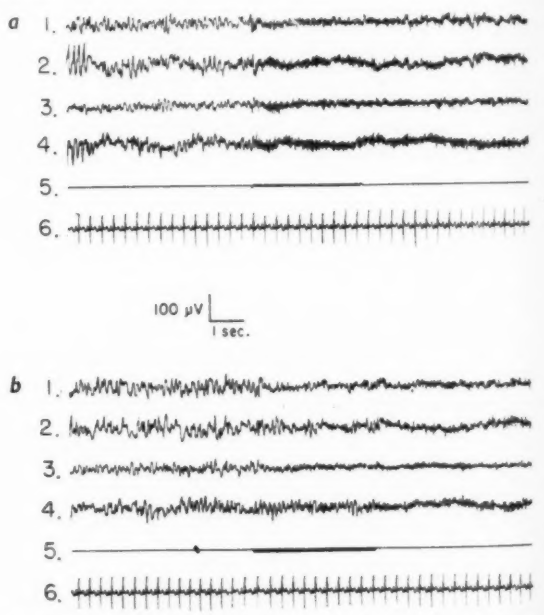

FIG. 2.—Electroencephalograms illustrating activation following: *a*, 3 sec. electrical stimulation of mid-brain reticular formation; *b*, tactile stimulation (stroking skin of back with glass rod). Note replacement of spontaneous slow potentials by low-voltage fast activity during and following each stimulus. Channels: **1**, left post. cruciate gyrus to left marginal gyrus; **2**, right post. cruciate gyrus to right marginal gyrus; **3**, left post. cruciate gyrus to right post. cruciate gyrus; **4**, left marginal gyrus to right marginal gyrus; **5**, stimulus marker; **6**, electrocardiogram.

The activated electroencephalographic pattern, the so-called "desynchronized," "asynchronous," or "alerted" pattern which is usually associated with behavioural arousal, is characterized by low-voltage fast activity (30 to 50 c./sec.) with reduction in voltage of the slow (1 to 10 c./sec.) waves seen in the drowsy or sleeping animal and, of course, an absence of sleep spindles. It must be realized, however, that various gradations in electroencephalographic pattern exist between the extremes of sleep and full arousal as has been emphasized by Rothballer (1956). Moreover, drugs may sometimes produce electroencephalographic patterns which simulate those of sleep or full arousal without corresponding overt behaviour (Wikler, 1952 ; Bradley and Elkes, 1957).

The drugs examined have been divided into groups according to their effects on other parts of the nervous system.

Anti-muscarinic Agents

Hyoscine Hydrobromide.—This is the most potent depressant of reticular activation of the electroencephalogram which we have tested. Fig. 3*a* shows part of a record taken about 5 min. after the intravenous injection of 0.1 mg./kg. of hyoscine. Cortical potentials were invariably slowed in frequency by the drug, and sleep spindles usually appeared. The almost complete abolition of the response to reticular stimulation will be noted. Tactile stimulation was likewise ineffective in promoting activation.

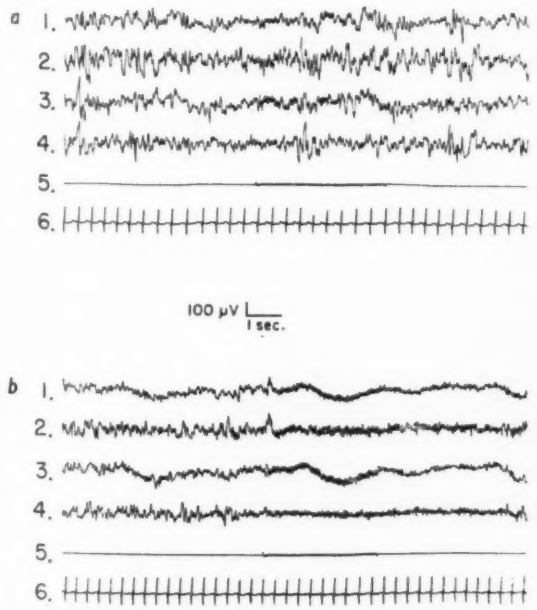

FIG. 3.—Electroencephalograms, channels as in Fig. 2. *a*, Failure of electroencephalographic activation by reticular stimulation 5 min. after giving intravenous hyoscine hydrobromide (0·1 mg./kg.); *b*, reversal of this hyoscine-block of reticular activating system by subsequent dose of physostigmine sulphate (0 2 mg./kg.).

Atropine Sulphate.—Compared with hyoscine, we found atropine to be relatively weak as a depressant of reticular activation. Doses exceeding 0.4 mg./kg. produced slowing of the spontaneous cortical potentials. In the dose range 2 to 10 mg./kg., activation occurred only during the period of application of the electrical stimulus ; even after 10 mg./kg., complete block of reticular activation, such as was seen with hyoscine, did not occur ; the response to tactile stimulation was, however, abolished at this dose.

Methanthelinium Bromide.—It was not expected that this quaternary ammonium compound would enter the brain, and it was therefore not surprising to find that amounts as large as 10 mg./kg. did not impair the response to reticular stimulation. A transient slowing of the electrocortical potentials occasionally followed the injection and seemed to be associated with the brief hypotensive effect of the drug.

An intracerebroventricular cannula (Feldberg and Sherwood, 1953) was inserted in one experiment and 5 mg. methanthelinium was injected. No changes in the spontaneous electrical activity or in the response to reticular stimulation occurred during the ensuing hour.

It is thus conceivable that a drug such as methanthelinium might be valuable for premedicating animals used in this type of work when atropine or hyoscine is contraindicated because of the central effects described above.

Reversal of Hyoscine Effects by Physostigmine

In animals treated with hyoscine (as in Fig. 3*a*), it was found possible to reverse the effects of the drug on the spontaneous cortical potentials and on reticular activation of the electroencephalogram with physostigmine sulphate (0.2 mg./kg.) (Fig. 3*b*). This would appear to offer the most striking evidence for the existence of a cholinergic type of transmission somewhere within the reticular activating system.

Neostigmine does not appear to exert central effects (Bradley and Elkes, 1957), probably because of its quaternary nature.

Anti-nicotinic Agents

The results with hyoscine, which blocks reticular activation in very low doses, point strongly towards a cholinergic mechanism in which acetylcholine, or some other choline ester, is exerting a muscarine-like action. It was therefore considered important to test three drugs which dis-

play anti-nicotinic effects on the peripheral nervous system and can also enter the central nervous system to see whether they were capable of blocking reticular activation of the electroencephalogram.

Nicotine.—50 mg./kg. of nicotine base was administered in divided doses to each of two cats, to see whether the secondary paralysing actions on ganglia and skeletal muscle were also manifested on the reticular activating system. After the initial cardiovascular effects had subsided, the electroencephalographic voltage was somewhat lower and the frequency higher than before. Occasional convulsive spikes appeared. However, clear signs of further electroencephalographic activation were obtainable on reticular stimulation (Fig. 4*a*).

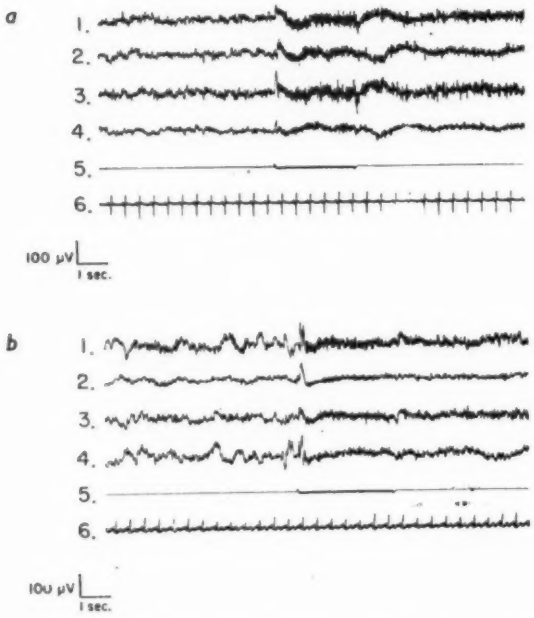

FIG. 4.—Electroencephalograms, channels as in Fig. 2. *a*, Low-voltage, moderately fast, spontaneous cortical potentials after intravenous nicotine base (50 mg./kg.). There is clear evidence of further activation on reticular stimulation. *b*, Failure of 10 mg./kg. mecamylamine hydrochloride to block reticular activation.

Mecamylamine Hydrochloride.—This potent ganglionic blocking agent, chemically a secondary amine, is known to enter the brain readily (Milne, Rowe, Somers, Muehrcke, and Crawford, 1957). Clinically, large doses may produce temporary psychoses or severe tremors (Harington and Kincaid-Smith, 1958).

We have given four cats intravenous injections of 10 mg./kg. mecamylamine and have been unable to demonstrate any change in the resting electroencephalographic pattern or in the activation produced by reticular stimulation (Fig. 4*b*), though ganglionic blockade was evident from a fall in blood pressure of about 30 mm. Hg.

Dihydro-β-erythroidine Hydrobromide.—This alkaloid has a well-known paralysing action on the skeletal neuromuscular junction which, in cats, is exerted in doses of about 0.5 mg./kg. It also blocks ganglionic transmission (Randall, 1951; Megirian, Leary, and Slater, 1955). Transmission in the spinal cord across the synapse between the motoneurone collateral and the Renshaw cell is readily blocked by intravenous injections of 0.1 mg./kg. (Eccles, Fatt, and Koketsu, 1954). Thus there is evidence that this alkaloid enters the central nervous system.

We have administered large intravenous doses of up to 20 mg./kg. of dihydro-β-erythroidine to three cats with no evidence of impairment of the response to reticular stimulation. The spontaneous electrocortical potentials were slowed only during the initial hypotensive response to the drug.

It is of interest to note that Starzl *et al.* (1951) used the parent alkaloid, β-erythroidine, for immobilizing animals during their experiments and did not mention any interference by the drug with electrocortical activity.

Thus we have no evidence from these experiments that the presumed cholinergic synapses in the ascending reticular system are influenced by drugs which display anti-nicotinic properties elsewhere in the nervous system.

Interneuronal Blocking Drugs

Previous investigators have shown that neither mephenesin (Domino, 1955; King, 1956) nor meprobamate (Gangloff, 1958) given in the usual doses depresses electrocortical activation following recticular stimulation. On the other hand barbiturates, which depress synapses in general, readily block such reticular activation (French *et al.*, 1953a).

Pentobarbitone Sodium.—Fig. 5*a* shows the typically slowed electrocortical potentials seen after 10 mg./kg. of pentobarbitone intravenously, illustrating failure of activation during or following reticular stimulation. In contradistinction to hyoscine, however, it was not possible to antagonize the blocking action of small doses of pentobarbitone by giving physostigmine. Nevertheless, 0.4 mg./kg. of the latter agent did increase the frequency of the spontaneous electrocortical potentials after pentobarbitone and caused a disappearance of sleep spindles (Fig. 5*b*).

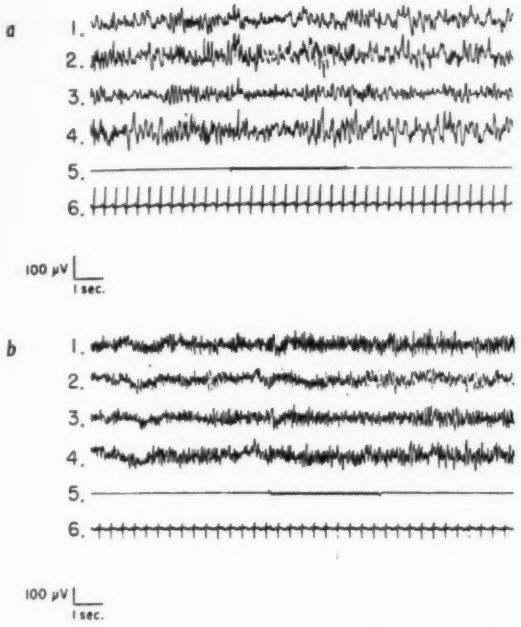

FIG. 5.—Electroencephalograms, channels as in Fig. 2. *a*, Typical failure of electrocortical activation after intravenous pentobarbitone (10 mg./kg.). *b*, Effect of 0·4 mg./kg. physostigmine on block of reticular activating system produced by pentobarbitone. Spontaneous electrical activity after pentobarbitone and physostigmine shows a greater component of fast activity than in *a*, but it will be seen that reticular stimulation in *b* produced no further activation.

Lignocaine Hydrochloride. — Bernhard and Bohm (1955) demonstrated some depression of spinal polysynaptic reflexes after intravenous lignocaine, and they suggested that the drug might also depress the bulbar reticular formation.

Fig. 6*a* shows a record taken from a cat which had been given 15 mg./kg. of lignocaine slowly enough to produce only a minor fall in blood pressure. The spontaneous potentials were immediately slowed and sleep spindles appeared. Electroencephalographic activation, however, readily occurred on reticular stimulation, though its duration following the stimulus was often shortened. Thus lignocaine, at this dose, appeared to be causing some depression of the reticular activating system. The effects of the drug persisted for only about 15 min.

It was found that the electroencephalographic effects of lignocaine, whilst they lasted, could be promptly abolished by giving physostigmine. This led us to consider the possibility of an atropine-like effect of lignocaine. We therefore tested the latter against the depressor responses to intravenous injections of 2 μg. of carbachol in a non-atropinized cat. Injections of 5 mg./kg. lignocaine intravenously abolished, for periods of about 1 min., the depressor effect of carbachol. The significance of this will be discussed later. It is of interest to note that Peterson (1955a) detected some anti-muscarinic activity in procaine.

Large injections of lignocaine (30 mg./kg.) resulted in severe hypotension and bradycardia, making it impossible to distinguish drug effects on the brain from ischaemic effects.

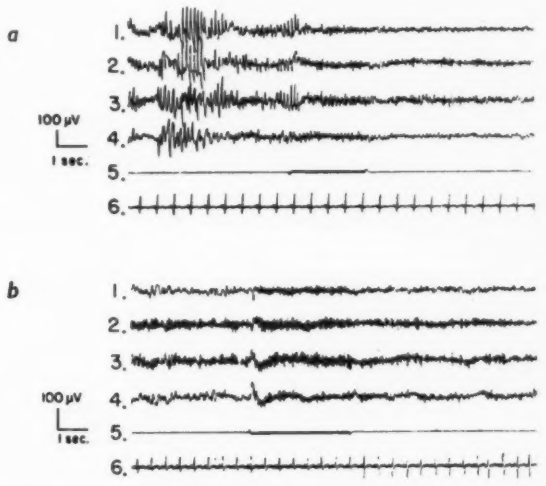

FIG. 6.—Electroencephalograms, channels as in Fig. 2. *a*, Cortical potentials following slow intravenous injection of lignocaine hydrochloride (15 mg./kg.). Slowed spontaneous activity with sleep spindles; electroencephalographic activation still evoked by reticular stimulation; duration of such activation usually reduced by lignocaine. *b*, Effect of dihydroergotamine methanesulphonate (1 mg./kg.). Spontaneous fast activity in channels 2 and 3, but clear signs of activation by reticular stimulation in channels 1 and 4.

Adrenergic Blocking Agents

The fact that adrenaline administered systemically can bring about electroencephalographic activation (Bonvallet *et al.*, 1954) has led many to suspect the existence of an adrenergic type of neurohumoral transmission within certain parts of the ascending reticular system. We have therefore tested some drugs which block peripheral adrenergic neuro-effector systems for possible actions on the reticular system.

Ergot Derivatives.—Neither ergotamine tartrate nor dihydroergotamine methanesulphonate, in amounts many times those producing full adrenaline-reversal, were capable of depressing reticular activation. The record of Fig. 6*b* was taken 10 min. after injecting 1 mg./kg. of the hydrogenated alkaloid; the spontaneous potentials tend to be fairly fast in frequency, but clear signs of further activation could be seen on reticular stimulation.

Phenoxybenzamine Hydrochloride.—Intravenous injections of this compound, given in divided amounts up to 10 mg./kg., resulted in slowing of the spontaneous cortical potentials, perhaps due to a concomitant fall in blood pressure to about 55 mm. Hg produced by the drug. Nevertheless, we were able to elicit clear-cut signs of reticular activation in the electroencephalogram.

Azapetine Phosphate.—This drug produced a smaller hypotensive reaction than did phenoxybenzamine and, in moderate doses, electroencephalographic activation was not prevented. Injections of 30 mg./kg. resulted in a convulsive type of electroencephalographic pattern from which it was not possible to discern the effects of reticular stimulation.

Choline 2 : 6-Xylyl Ether Bromide.—The adrenergic blockade produced by this drug (Hey and Willey, 1954) differs fundamentally from that of the previously mentioned compounds, since it reduces the amount of adrenergic transmitter released (Exley, 1957); this may be due to interference with the biosynthesis of the mediator (Exley, 1956; Coupland and Exley, 1957; Bain and Fielden, 1957).

Intravenous injections of 10 mg./kg. of the drug failed to modify either the spontaneous electroencephalogram or the response to reticular stimulation. Being a quaternary compound, it probably failed to enter the central nervous system. However, an injection of 2.5 mg. directly into the right cerebral ventricle lilkewise failed to produce any change.

The adrenergic blocking drugs as a whole therefore do not seem to exert any marked action on the reticular activating system.

Discussion

Evidence for the participation of a cholinergic mechanism at some point within the ascending reticular system is indirect, and has been well reviewed by Himwich and Rinaldi (1957). In particular, they have demonstrated electroencephalographic activation in rabbits following intracarotid injection of either acetylcholine or dyflos, and have further shown that atropine can both reverse these effects and block the activation following electrical stimulation of the reticular formation. Many of the antiparkinson drugs also block the effects of reticular stimulation (Rinaldi and Himwich, 1955c).

We were interested in trying to determine whether the action of the hypothetical cholinergic transmitter within the ascending reticular system was muscarine-like or nicotine-like. To some extent we have succeeded in the attempt, since we have found that the anti-nicotinic agents dihydro-β-erythroidine and mecamylamine, and large doses of nicotine itself, certainly fail to block reticular activation of the electroencephalogram. It is relevant that the nicotinic cholinergic type of synapse between the motorneurone collateral and the Renshaw cell of the cord can be readily blocked by dihydro-β-erythroidine (Eccles *et al.*, 1954). This synapse therefore seems to differ, at least pharmacologically, from the inferred cholinergic synapses associated with the reticular activating system.

On the other hand, it seems that the hypothetical cholinergic transmitter, occurring somewhere in the reticular activating system, displays properties analogous in some respects to the muscarinic actions of acetylcholine. We have confirmed the observation of Rinaldi and Himwich (1955a) that atropine depresses the reticular activating system. More recently, Kuehn and Schallek (1958) have reported that hyoscine is about one hundred times as potent as atropine in this respect. We have also found a high order of blocking activity in hyoscine, and have demonstrated a clear antagonism of this blockade by physostigmine. The marked difference in potency between atropine and hyoscine is, however, surprising and difficult to explain in the light of the moderate difference of about twofold in the peripheral anti-muscarinic activities of the two alkaloids. That hyoscine has marked central depressant effects, not seen clinically with atropine, is too well known for elaboration here; it is a salient example of the profound pharmacological change that can result from a slight alteration in chemical structure.

The site of the depressant action of hyoscine on the reticular activating system has not been established; Bradley and Key (1958) believe it to be at higher levels than the mid-brain reticular formation, possibly within the diffuse thalamic projection. If hyoscine were acting on the reticular formation itself, then the animal presumably would exhibit block of reticular activation of the electroencephalogram together with behavioural somnolence, as do cats with induced lesions of the rostral mid-brain reticular substance (Lindsley, Schreiner, Knowles, and Magoun, 1950). But Bradley and Elkes (1957) have clearly shown that hyoscine does not enforce behavioural sleep in cats.

It is not easy to explain the differences in the reactions of the various central and peripheral synapses to the interneuronal blocking drugs and

barbiturates. Reference has already been made to the failure of mephenesin or meprobamate to influence the reticular activating system, in spite of their depressant actions on the polysynaptic spinal pathways. Local anaesthetics, such as procaine (Peterson, 1955b) or lignocaine (Bernhard and Bohm, 1955), are capable of depressing the cord reflexes ; in high concentrations they may also depress transmission in sympathetic ganglia. We have found that moderate doses of lignocaine invariably slow the spontaneous electrical activity, cause the appearance of sleep spindles and may reduce the duration of electroencephalographic activation following reticular stimulation. The fact that physostigmine can promptly reverse these effects suggests to us that the mode of action of lignocaine on the reticular activating system might resemble that of atropine more closely than that of pentobarbitone. Our demonstration of a very transitory anti-muscarinic effect of lignocaine on the cardiovascular system raises the possibility that the drug may fix more firmly to structures in the central nervous system, and hence produce atropine-like effects of longer duration than it does peripherally. If the central action is like that of atropine or hyoscine then lignocaine would not be expected to cause behavioural sleep in unrestrained animals.

The barbiturates may be considered to show pharmacological actions on the nervous system which to some extent overlap those of the anti-muscarinic, anti-nicotinic, and interneuronal blocking agents. Barbiturates depress reticular activation, ganglionic transmission, and the cord reflexes, but they do not have peripheral atropine-like effects (Exley, 1954) or skeletal neuromuscular-blocking effects (Quilliam, 1955). Our observation that depression of reticular activation by pentobarbitone cannot be overcome with physostigmine is in keeping with the fact that barbiturate narcosis cannot be antagonized with physostigmine in the unrestrained cat (Bradley and Elkes, 1957).

In conclusion, it would seem that present evidence for a cholinergic type of interneuronal transmission within the reticular activating system is more abundant than that for an adrenergic process. Admittedly, catechol amines are found in brain tissue, and injected adrenaline may under certain circumstances activate the electroencephalogram, but these facts can only provide suggestive evidence of an extremely indirect nature. There are some facts which are in direct opposition to such an adrenergic transmission concept. First, reserpine depletes brain tissue of its noradrenaline content (Brodie, Olin, Kuntzman, and Shore, 1957), but moderate to high doses of reserpine do not appear to block activation of the electroencephalogram by reticular stimulation (Rinaldi and Himwich, 1955d ; Killam and Killam, 1956) ; second, Kuehn and Schallek (1958) have been unable to demonstrate any change in stimulus threshold for reticular activation after treatment of cats with the amine oxidase inhibitor iproniazid ; and, third, we have been unable to detect depressant actions on the reticular activating system in five different adrenergic blocking drugs.

Further elucidation of the problems of central synaptic transmission still awaits unequivocal experiments demonstrating the liberation of specific transmitters following controlled central nervous activity. Meanwhile, however, neuropharmacological approaches can offer much in the way of logical elimination.

Our thanks go to Dr. Raymond N. Bieter for his kindness in reading the manuscript, and to Dr. J. C. Richards, of Merck Sharp and Dohme Research Laboratories, for a gift of dihydro-β-erythroidine. This work was undertaken during the tenure by one of us [M. C. F.] of a Post-Doctoral Fellowship in Neuropharmacology.

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A MICROBATH

BY

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A microbath, with a volume of less than 50 cubic millimetres, for testing the effects of drugs on small pieces of smooth muscle, is described. Drugs are applied automatically in any desired order and the record of their effects is amplified electrically. In this apparatus the response of small pieces of rat uterus to drugs is apt to be nearly all-or-none. Contractions of the muscle from a single ring of guinea-pig trachea were recorded. A fall of *p*H was found to reduce the effect of 5-hydroxytryptamine on this muscle while leaving the effects of tryptamine and acetylcholine unchanged.

Those who wish to detect small amounts of drug by their effects on isolated smooth muscle generally use small baths in order to increase the concentration produced by a given total dose of drug. However, it is generally not convenient, with the usual type of bath, to diminish the volume below about 2 ml. If the muscle is suspended in air, drugs may be applied to the surface of the muscle, as in the method known as superfusion (Gaddum, 1953) where the salt solution normally flows continuously over the muscle and is stopped when drugs are applied. This system has been found satisfactory for certain purposes but has various disadvantages. The volume of salt solution is probably about as small as is possible with large pieces of muscle, but the technique does not lend itself to the use of small pieces of muscle. The mechanical effects of each drop then become important. If the suspended muscle is fixed horizontally the fluid may be drained away through a tapered tube fixed under the muscle without forming drops, but it is then difficult to adjust the apparatus satisfactorily. Attempts to overcome this problem have led to the use of the small baths through which fluid flows continuously. These had a volume of 25 to 35 c.mm. which is equal to that of a small drop, but smaller baths on the same principle could probably be made.

METHODS

The apparatus is shown in Fig. 1. The bath is a horizontal one. It consists of a hole (diameter 1.7 mm.) drilled parallel with the axis through a piece of Perspex rod (diameter 2 cm., length 1.5 cm.) so that it just does not reach the opposite face. It is completed by a smaller hole just large enough to take the smallest available sewing needle. Near this small hole a series of radial holes are drilled (diameter 0.8 mm.) through which various solutions may be made to flow. At the far end of the bath the fluid runs vertically down a narrow groove on a flat Perspex surface without forming drops. The fluid is collected on a shelf so that it runs back through a larger hole in the Perspex rod over the bulb of a thermometer and then out through a hole in the side of a small rod of Perspex fixed below the bath, so that the fluid again runs over a vertical Perspex surface before eventually forming drops.

One bath was made with annular platinum electrodes near each end so that the muscle could be stimulated electrically when desired.

A brass rod is screwed into the side of the bath to hold it in position. Before reaching the bath the fluids may be warmed in glass tubes fixed to a Perspex plate. This plate and the bath are both attached to a horizontal brass rod which can be moved in the direction of its own length by a rack and pinion. The fluids run from the glass tubes to the bath through fine polythene tubing pulled to a point and inserted directly into the radial holes in the bath.

The temperature is maintained by an infra-red electric lamp above the apparatus the position of which is adjusted to give the desired temperature on the thermometer in the bath and the smallest possible changes when the fluids are changed; this was found to mean that the lamp was nearer the bath than the warming coils. In some early experiments, bubbles formed in the bath; this complication was avoided when the solutions were equilibrated with air at a temperature of about 40 to 45° and then cooled before the experiment started.

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Drugs can be applied to the muscle with a small syringe through one of the radial holes in the Perspex rod, after stopping the flow of saline. A volume of 0.25 ml. is sufficient to replace all the fluid in the bath 7 to 10 times, and smaller volumes can be used.

The drug solution is left in the bath for a fixed time and then washed out by starting the flow of saline again. The syringe needle is left in the hole until this stage, in order to avoid sucking drug back into the hole when the syringe is removed.

Drugs can also be applied mechanically according to a prearranged schedule. Four different drug solutions are held in glass tubes fixed in a stand about 20 cm. above the bath. The flow from these is controlled by relays similar to those used by Schild (1942). The solutions then run through glass tubes on the Perspex plate by the side of the tube which warms the main solution. They then flow through fine polythene tubing to four different radial holes in the Perspex bath.

The relays are controlled by a timing device similar to that described by Austin (see Adam, Hardwick, and Spencer, 1954). The first timing circuit controls the flow of drug at the bath which lasts a few seconds. The second circuit controls the duration of its stay in the bath. The third is not normally used. The fourth and fifth control the flow of salt solution during the interval between contractions. This cycle of events is timed on a stopwatch which is automatically started from zero when each dose of drug is applied.

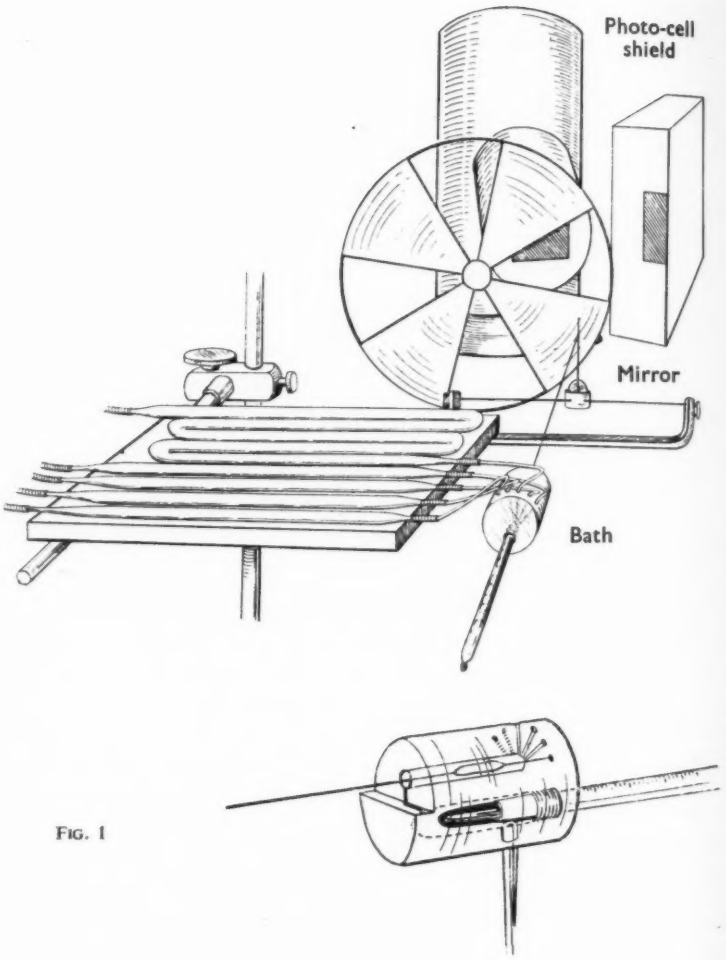

FIG. 1

The order in which the drugs are given is controlled by a set of 24 six-way switches which are connected with 24 positions on a post office uniselector, so that each switch is used in turn. The first four positions of each switch are connected with the four relays which control the solutions. The fifth is connected with nothing at all, so that drugs may be omitted, and the sixth position is connected so that the selector jumps past that position. The apparatus thus allows up to 4 drug solutions to be automatically applied in any order to produce a repeating series of up to 24 doses.

Fine threads are obtained by untwisting terylene gossamer thread and warming to remove the twist. These are attached to each end of a small piece of muscle (5 to 10 mm. in length); one of these is threaded with a needle through the small hole at the end of the bath; the muscle is pulled into position in the bath and the thread fixed with plasticine; the other end is attached to a lever. The most satisfactory results have been obtained with a torsion lever. Isotonic levers tend to be unreliable since a very small frictional resistance is enough to restrict the free movement of these small pieces of muscle. The torsion lever is similar to that described by Condon (1957) and consists of 11 cm. of thin steel wire in a holder which allows the tension in the wire, and the resting position of the lever to be independently adjusted. The thread from the muscle can be attached at different distances from the wire; sometimes the muscle contractions have been nearly isometric, but usually the muscle has been allowed to shorten appreciably. A concave mirror is fixed to the torsion wire to record its movements by altering the amount of light falling on a photocell (G.E.C. gas-filled potassium cell). The filament of a lamp (24 watts, 24 v. D.C.) is focused on to the concave mirror which in turn focuses the image of a square diaphragm on to the photocell. A 108 v. battery is connected across the photocell with a resistance of

2 megohms in series; the voltage changes across the resistance are led, through a capacitance, to an A.C. amplifier. The light beam is interrupted by a rotating sector disc so that the output of the photocell fluctuates and provides a suitable input for the A.C. amplifier. The photocell is screened as much as possible, but some stray light inevitably falls on it. When the rotating disk is placed immediately in front of the photocell this stray light is interrupted and contributes to the record. If, however, the light beam is interrupted between the lamp and the mirror, stray light falls on the photocell without interruption and, provided the photocell is not saturated, the A.C. component of its output is not much affected. The amplified current is rectified and fed to a pen recorder writing on slow-moving kymograph paper. A small 50 cycle A.C. voltage is added to the rectified amplifier output; this imparts a small vibration to the pen which diminishes the effects of friction. When too much vibration was applied a curious artefact appeared in which the pen wandered with a slow frequency over the paper producing a record which seemed, at first, to be due to spontaneous movements of the muscle. This artefact was partly due to the use of a steel pen whose end was not quite smooth and was reduced when the actual writing point was polythene.

Flexibility in operation is obtained by using a short optical lever and relying on electrical amplification to provide most of the magnification of small contractions. A long optical lever provides additional sensitivity, but larger contractions then move the light right off the photocell. The length of the optical lever is largely determined by the radius of the concave mirror. We use a mirror with a radius of 15 cm. and a condenser lens with a focal length of 5.5 cm. The mirror is about 10 cm. from the square diaphragm and about 30 cm. from the photocell. With the thread attached 3 cm. from the torsion wire the optical magnification is 10. The photocell is 3 cm. long so that a 3 mm. contraction of the muscle can be recorded. With maximum amplification, however, the full range of the recorder (about 10 cm.) is covered by a contraction of 0.03 mm.

Results

When the continuous flow of fluid was interrupted the temperature recorded on the thermometer was liable to alter by about 1° and this sometimes affected the behaviour of the muscle. In many cases, for example, there was a temporary increase of temperature when the flow was resumed, which sometimes caused a contraction of the uterus and a relaxation of the trachea. In order to detect errors due to this cause drug-free solutions were often tested and, when the automatic apparatus was used, were usually alternated with each application of drug. It was generally possible by adjusting the position of the heating lamp to avoid serious interference due to this effect.

On several occasions loss of activity of dilute 5-hydroxytryptamine solutions was observed. This was first seen when polythene tubes were used to warm the solutions and disappeared when these were replaced by glass tubes. Something similar happened later and was shown to be due to destruction of 5-hydroxytryptamine by micro-organisms growing in the small diameter rubber tubing connecting the drug reservoir to the warming tubes.

Rat Uterus.—Small pieces of longitudinal muscle of the uterus of rats have been used. They were generally cut from the surface by hand with a fine pair of scissors in one cut so that they were slightly tapered at the ends. These were about 5 mm. long and 0.5 mm. wide. They were suspended in de Jalon solution (Gaddum, Peart, and Vogt, 1949) which had been equilibrated with air at 45°. They contracted in the presence of 5-hydroxytryptamine and carbachol in concentrations similar to those used in larger baths and, in good conditions, gave regular results. If doses were given too frequently tachyphylaxis to both carbachol and 5-hydroxytryptamine developed. The preparations were particularly liable to give all-or-none results, so that one dose had no apparent effect and a dose 50% larger had a nearly maximum effect (Fig. 2).

When the solutions were equilibrated with a mixture of 5% CO_2 in oxygen the effects of 5-hydroxytryptamine and carbachol were reduced (Fig. 3).

Guinea-pig Trachea.—Castillo and de Beer (1947), who introduced the use of chains made by tying pieces of guinea-pig trachea together, give some references to earlier work with tracheal muscle.

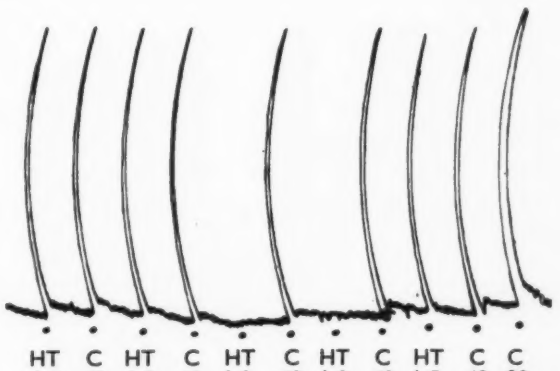

FIG. 2.—Rat uterus, 30°. Drug cycle, 3½ min. HT, 5-hydroxytryptamine; C, carbachol. Concentrations are given in mg./100 l. Blank control between each dose. A steep dose-effect curve is seen with 5-hydroxytryptamine.

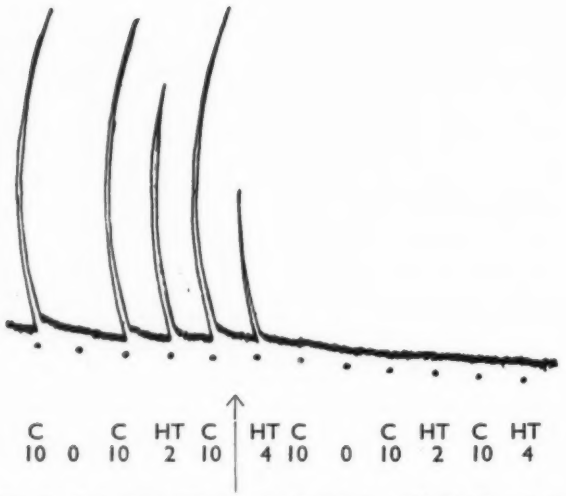

FIG. 3.—Rat uterus, 25°. Drug cycle, 3½ min. HT, 5-hydroxytryptamine; C, carbachol. Concentrations, mg./100 l. de Jalon solution equilibrated with air; from arrow the solution was equilibrated with 5% CO_2 and 95% O_2. Effects depressed.

In the present experiments, preparations were made from a single ring of guinea-pig trachea. The ring was opened by cutting through the cartilage at a point directly opposite the strip of muscle; thread was tied round each piece of cartilage about half-way between the muscle and the cut end. As much as possible of the cartilage was then cut off, but some cartilage remained between the ligatures so that the preparation was not quite flat. When the thread was tied closer to the muscle the preparation was flatter but was less sensitive to drugs. In some later experiments, the diameter of the bath was increased to accommodate the cartilage.

In the early experiments the bathing fluid was Tyrode solution, but in later experiments more satisfactory results were obtained with Locke solution containing 0.1% (instead of 0.015%) $NaHCO_3$ and 0.8% NaCl.

In experiments with Tyrode solution equilibrated with air at 40 to 45° sensitivity was slow to develop and the preparations were often set up in the evening for use the next day. Fig. 4 shows that histamine caused a contraction in a concentration of 10 mg./100 l.

When air is bubbled through such solutions the *p*H is commonly greater than 8. In these conditions the tone of the preparation was apt to increase. This increase could sometimes be stopped by lowering the temperature, and was found to be depressed when the bathing fluid was made more acid either by adding ascorbic acid or by bubbling a mixture of 5% CO_2 in oxygen

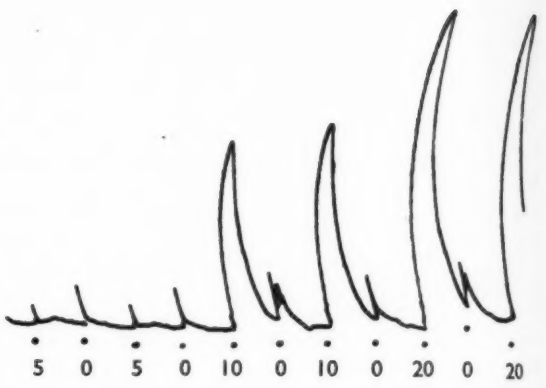

FIG. 4.—Guinea-pig trachea. Tyrode, 26°. Drug cycle, 8½ min. with controls between. Drug contact, 104 sec. Histamine concentrations in mg./100 l. are shown on the abscissa.

through the fluid at 40 to 45° before use. The Locke solution used in the later experiments was therefore generally treated with CO_2 in this way. In these conditions the muscle responded to drugs even in the early stages of the experiment though it was still generally more sensitive on the second day.

Making the solution more acid had the effect of reducing the response to 5-hydroxytryptamine, but it had comparatively little effect on the responses to tryptamine and acetylcholine (Fig. 5).

Preparations made from one particular guinea-pig showed regular bursts of rhythmic activity which were inhibited by exposure to a small dose of adrenaline for a short time (Fig. 6). Atropine had no effect, except in high concentrations (4×10^{-7}) perfused continuously, which reduced the frequency of the bursts.

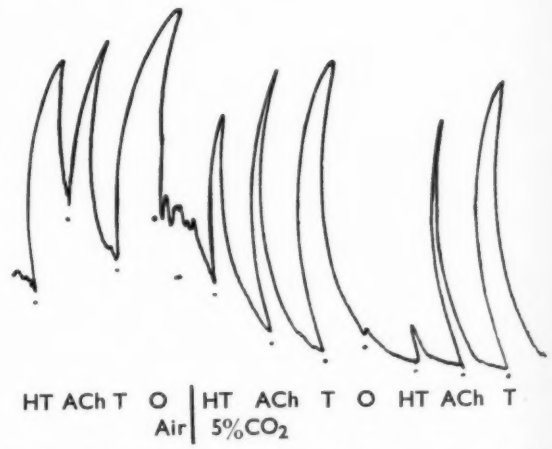

FIG. 5.—Guinea-pig trachea. Tyrode, 30°. Drug cycle, 4¾ min. HT, 5-hydroxytryptamine, 0.1 μg./ml.; ACh, acetylcholine, 0.1 μg./ml.; T, tryptamine, 40 μg./ml.

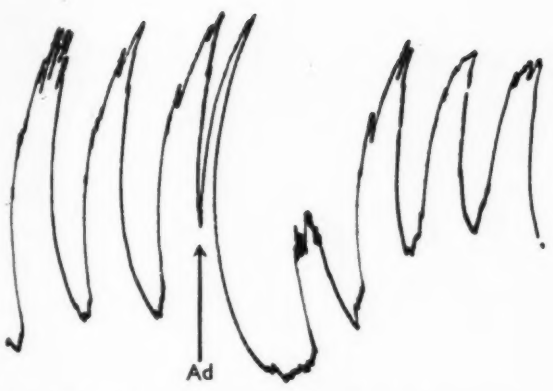

FIG. 6.—Guinea-pig trachea. Tyrode, 26°. Spontaneous movements depressed by adrenaline (Ad) 100 mg./l. for 30 sec.

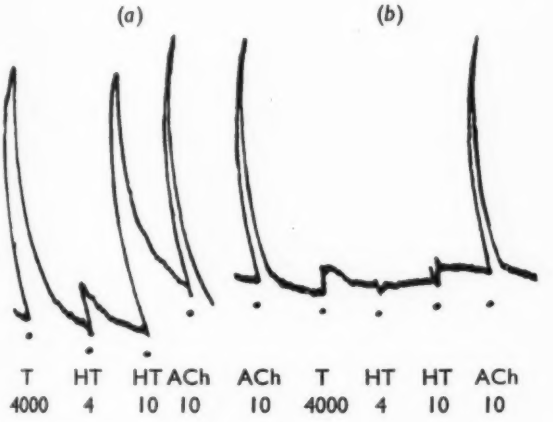

FIG. 7.—Guinea-pig trachea. Tyrode. Drug cycle, 4 min. Drug contact, 35 sec. T, Tryptamine; HT, 5-hydroxytryptamine; ACh, Acetylcholine. Concentrations are shown in mg./100 l. Between (*a*) and (*b*) the muscle was exposed to 2-bromolysergic acid diethylamide (BOL) for 45 min. Effects of T and HT were depressed.

Fig. 7 shows that the response to 5-hydroxytryptamine and tryptamine was abolished by 2-bromolysergic acid diethylamide while the response to acetylcholine was unchanged. The effect of acetylcholine (2×10^{-7}) on another preparation was abolished by atropine (10^{-8}).

DISCUSSION

This apparatus is suitable for studying the responses of small pieces of plain muscle which might otherwise be difficult to use. It might perhaps be suitable for the hearts of molluscs which are sometimes liable to make records on smoked paper which cannot easily be reproduced (Gaddum and Paasonen, 1955). The pen-writer produces clear records of almost any desired size.

It might perhaps be used in experiments such as those of Gaddum, Jang, and Kwiatkowski (1939) where the fluid from a perfused tissue runs continuously over a piece of plain muscle and substances liberated by stimulating a nerve in the tissue are detected as they flow over the muscle.

We are much indebted to Dr. D. C. Simpson for his advice and help in making the recording apparatus.

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THE DIFFERENCE IN THE METABOLISM OF INJECTED [^{14}C]HISTAMINE IN MALE AND FEMALE RATS

BY

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(RECEIVED AUGUST 29, 1958)

[^{14}C]Histamine was injected subcutaneously in rats. The urine was collected for 24 hr. and analysed for [^{14}C]histamine and its various metabolites. It was found that male rats excreted less unchanged [^{14}C]histamine than female ones, the difference between the sexes existing also when the animals were treated with aminoguanidine. The cause of the sexual difference appeared to be that the male rats had a larger capacity to methylate histamine.

Leitch, Debley, and Haley (1956) observed that male rats excreted about 10 times less histamine in the urine than female ones. Similar results were obtained by Gustafsson, Kahlson, and Rosengren (1957), who found that males excreted much less free histamine than females.

The urinary excretion of histamine, particularly in rats, has been used as an indicator of liberation or production of histamine in the body (see, for example, Wilson, 1954; Schayer, Davis, and Smiley, 1955; Leitch *et al.*, 1956). It appeared therefore of interest to study more closely the cause of the difference in histamine excretion between the sexes. The smaller urinary histamine excretion in male rats could be due to many causes. *A priori*, it seems most likely that it is due either to a lower rate of production or to a more efficient metabolic transformation of histamine. The latter possibility was examined in the experiments described below.

METHODS

White rats, 4 to 8 months old, were used. They were of the same inbred stock as the animals used by Gustafsson *et al.* (1957) and Kahlson, Rosengren, and Westling (1958). The rats were given a synthetic, histamine-free diet and were kept in metabolism cages as described by Kahlson *et al.* (1958). Urine was collected for 24 hr. in a vessel containing hydrochloric acid so as to bring the *p*H of the collected urine to below 2.

Histamine labelled with ^{14}C in the 2-position of the imidazole ring (The Radiochemical Centre, Amersham, England) was injected subcutaneously in a dose of 0.03 to 0.16 mg./kg. body weight. Aminoguanidine sulphate (Eastman Organic Chemicals, Rochester, U.S.A.) was injected subcutaneously in a dose of 20 mg./kg. twice daily.

Two rats were injected with [^{14}C]histamine twice, with an interval of three weeks; at one of the injections the rats were also treated with aminoguanidine.

The urine was assayed for total ^{14}C content with the procedure described by Schayer and Cooper (1956). The urinary content of [^{14}C]histamine and its various radioactive metabolites was measured with the isotope dilution techniques developed in recent years by Schayer and his colleagues. The following metabolites of [^{14}C]histamine were studied: methylhistamine {4-(2-aminoethyl)-1-methyl-imidazole}, methylimidazoleacetic acid (1-methylimidazol-4-ylacetic acid), imidazoleacetic acid (imidazol-4 (or 5)-ylacetic acid), and acetylhistamine. Histamine and the various metabolites were used as carriers in amounts equivalent to 206 or 412 mg. of the respective picrates. Imidazoleacetic acid was determined after hydrolysis of the urine in 1 N-HCl in a sealed glass tube at 160° for 5 hr. The values given for imidazoleacetic acid are therefore considered to include also conjugates (1-ribosylimidazol-4-ylacetic acid). The methods used were the same as described by Lindell and Schayer (1958). The estimations of methylimidazoleacetic acid and imidazoleacetic acid were made in duplicates, which agreed well.

^{14}C radioactivity was measured at infinite thickness under standardized conditions in a flow counter with a background of 21 to 25 counts/min.

RESULTS

Table I gives the amount of [^{14}C]histamine excreted, and it may be seen that the male rats excreted considerably less unmetabolized [^{14}C] histamine than the females. If the rats were given aminoguanidine sulphate, in a dosage which inhibits the action of histaminase (diamine oxidase)

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almost completely (Schayer, Kennedy, and Smiley, 1953), a larger proportion of the injected [^{14}C]histamine was excreted unchanged but the differences between the sexes persisted.

TABLE I

URINARY EXCRETION OF UNCHANGED [^{14}C]HISTAMINE DURING THE 24 HR. FOLLOWING A SUBCUTANEOUS INJECTION IN MALE AND FEMALE RATS AND AFTER INJECTION SUBCUTANEOUSLY WITH AMINOGUANIDINE SULPHATE

Two experiments were made on Rat No. 2 and 6. + = 20 mg./kg. of aminoguanidine was injected twice daily.

	Males					Females				
Rat No. ..	1	2	2	3	4	5	6	6	7	8
Body weight (g.)	250	320	315	415	250	230	210	225	185	265
Aminoguanidine treatment ..			+	+	+			+	+	+
[^{14}C]Histamine injected (mg./kg.) ..	0·15	0·16	0·16	0·03	0·15	0·15	0·15	0·16	0·05	0·05
[^{14}C]Histamine excreted (% of injected amount)	<2	1	3	2	<5	10	12	33	36	33

Table II gives the results of experiments in which some metabolites of [^{14}C]histamine were measured, and it may be seen that the relative amount of imidazoleacetic acid was about the same in males and females. In animals not given aminoguanidine, the total urinary imidazoleacetic acid accounted for slightly more than 50% of the injected [^{14}C]. With aminoguanidine treatment, this value decreased to 7 to 9%.

TABLE II

URINARY EXCRETION OF [^{14}C]HISTAMINE AND SOME OF ITS METABOLITES DURING THE 24 HR. FOLLOWING A SUBCUTANEOUS INJECTION OF [^{14}C]HISTAMINE

The values are expressed as % of the amount of ^{14}C injected and were obtained in the same experiments reported in Table I. For explanation of +, see Table I.

	Males			Female	
Rat No...	2	2	3	6	6
Aminoguanidine treatment ..		+	+		+
Histamine	1	3	2	12	33
Imidazoleacetic acid	51	9	7	51	7
Methylhistamine	16	41	34	4	17
Methylimidazoleacetic acid ..	7	17	22	6	14
Acetylhistamine	2	3	4	3	3
Total ..	77%	73%	69%	76%	74%

In the male rats methylation of [^{14}C]histamine, with consequent excretion of methylhistamine and its oxidation product, methylimidazoleacetic acid, was more important than in the females. In males given aminoguanidine, methylation of histamine (indicated by excretion of methylhistamine and methylimidazoleacetic acid) accounted for 56 and 58% of the injected [^{14}C].

Acetylation seemed to be of little importance in the metabolism of injected [^{14}C]histamine. Acetylhistamine accounted for only a few per cent. of the injected ^{14}C both in males and females, without or with aminoguanidine treatment.

Nearly all of the injected [^{14}C]histamine (87 to 104%) appeared in the urine during the 24 hr. collection period. All of the injected or excreted ^{14}C could, however, not be accounted for in terms of the radioactive compounds measured, which when added together constituted 69 to 76% of the injected ^{14}C. This result is similar to those obtained in men by Schayer and Cooper (1956) and in women by Lindell, Nilsson, Schayer, and Westling (unpublished observations). The failure to account for all [^{14}C] may have several explanations, but the possibility obviously exists that unknown metabolites may be present.

DISCUSSION

Under the prevailing experimental conditions male rats metabolized injected [^{14}C]histamine more efficiently than female ones. The greater efficiency was not connected with histaminase (diamine oxidase), since the expected product of the action of this enzyme, imidazoleacetic acid, occurred in equal amounts in males and females. Moreover, the difference between the sexes persisted under treatment with aminoguanidine, a powerful inhibitor of diamine oxidase (Schuler, 1952; Schayer *et al.*, 1953). The difference between the sexes in the metabolism of injected [^{14}C]histamine appears largely to be due to a greater capacity of the male rat to methylate histamine.

The observation of a more efficient histamine degradation in male rats gives a satisfactory explanation for the smaller excretion of free histamine in this sex (Leitch *et al.*, 1956; Gustafsson *et al.*, 1957), provided that histamine liberated in the body undergoes essentially the same metabolism as injected [^{14}C]histamine. This assumption receives some support from a comparison between the proportion of injected [^{14}C]histamine excreted unchanged and the amount of free urinary histamine excreted by the rats when they were not injected with [^{14}C]histamine. The 24 hr. excretion of free histamine (expressed as histamine base and determined by bioassay) in these rats was very roughly in the males 1 μg. (3 μg. with aminoguanidine) and in the females 20 μg. (80 μg. with aminoguanidine). The parallelism between these estimates and the proportion of injected [^{14}C]histamine excreted unchanged in

males and females, without and with aminoguanidine, makes it tempting to assume that the total daily liberation of histamine in male and female rats is about the same and that the fraction of this histamine escaping into the urine is determined by the capacity of the metabolizing enzymes. Direct evidence on this point is, however, at present lacking.

The finding of imidazoleacetic acid as the most important metabolite confirms the conclusion of Schayer (1953), that histaminase (diamine oxidase) is the most important histamine-metabolizing enzyme in rats. Earlier reports (Schayer *et al.*, 1953; Schayer *et al.*, 1955) indicated that, after inhibition of the histaminase in rats by aminoguanidine, the greater part of liberated or injected histamine came out unchanged in the urine. In the present experiments, even in female rats, not more than about one third of injected [^{14}C]histamine was excreted unchanged. The different results are likely to be due to the fact that, in the earlier studies which were made principally with paper chromatography, [^{14}C]methylhistamine gave spuriously high values for [^{14}C]histamine, a finding which has been discussed by Schayer and Cooper (1956).

It appears that the female rat is more suitable than the male for studies with the urinary excretion of histamine as an indicator of production or liberation of histamine in the body. Particularly so if aminoguanidine is given and precautions taken to diminish the contribution to the urinary excretion by histamine in the food and by histamine produced by intestinal bacteria (see Schayer *et al.*, 1955, and Kahlson *et al.*, 1958).

I am grateful to Dr. Richard W. Schayer, who during a stay at this Institute generously instructed me in his methods. Skilful technical assistance was given by Miss M.-B. Johansson.

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THE PHARMACOLOGICAL ACTIONS OF PEMPIDINE AND ITS ETHYL HOMOLOGUE

BY

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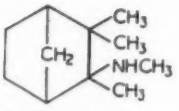
Pempidine, and other highly active ganglion blocking agents of the polyalkylpiperidine series, were developed from tertiary alkylamines, themselves weakly active, on the hypothesis that high activity was conferred by the presence in the molecule of a sterically hindered secondary or tertiary nitrogen atom. Pempidine and its N-ethyl homologue (26539) resembled mecamylamine qualitatively. All three drugs blocked sympathetic and parasympathetic ganglia; this action was slow in onset and protracted. They blocked neuromuscular transmission, but only about one hundredth as powerfully as ganglionic transmission. They caused a fall in amplitude and rate of the isolated heart, and reduced coronary flow. They had local anaesthetic properties in one of four tests used. They caused tremor. All were well absorbed when administered orally. Pempidine was about twice as active as mecamylamine on ganglia, but only about one half to one quarter as toxic as judged by death, growth, induction of tremor, or cardiotoxicity. Compound 26539 was also quantitatively superior to mecamylamine in respect of these safety margins, but unlike pempidine or mecamylamine damaged the pituitary gland and testis when administered daily for several months. The mode of action of the three drugs is discussed: the results give tentative support for the hypothesis that their action is intracellular.

We have recently reported the discovery of powerful ganglion-blocking and hypotensive properties in a series of polyalkylpiperidines (Spinks and Young, 1958). The most promising of these compounds were 1:2:2:6:6-pentamethylpiperidine (pempidine; Tenormal (I.C.I.) is the hydrogen tartrate) and 1-ethyl-2:2:6:6-tetramethylpiperidine (referred to subsequently by our code number, 26539). Lee, Wragg, Corne, Edge, and Reading (1958) have since reported their discovery independently of activity in the same series, and an account of the action of pempidine hydrogen tartrate in man has been given by Harrington, Kincaid-Smith, and Milne (1958).

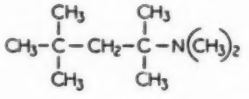
The object of this report is to describe our development of the piperidines, which was different from that of Lee *et al.* (1958), and to give a complete account of our pharmacological and toxicological studies of pempidine and 26539.

During the past five years we have been searching for ganglion-blocking activity among a wide variety of novel compounds; about 2,400 have been tested. Most of them were non-quaternary bases, since it was considered that a highly promising drug should be completely and regularly absorbed when given by mouth, and would almost certainly prove to be non-quaternary. The discovery of the secondary amine, mecamylamine, or 3-methylaminoisocamphane, by Stone, Torchiana, Navarro, and Beyer (1956) first showed that powerful ganglion-blocking activity could indeed be displayed by a non-quaternary base, and that it could be combined with excellent absorption from the gastrointestinal tract. On the other hand, it has become clear that the non-quaternary character of mecamylamine also enables it to enter the central nervous system and elicit side-effects such as tremor, hyper-reflexia, and, possibly, anxiety (Schneckloth, Corcoran, Dustan, and Page, 1956; Perry and Schroeder, 1957; Milne, Rowe, Somers, Muehrcke, and Crawford, 1957; Harrington and Kincaid-Smith, 1958) which, hitherto, have not been reported with quaternary ganglion-blocking agents. Like all ganglion-blocking drugs, mecamylamine also displays side-effects arising from its action on parasympathetic ganglia, and perhaps also from direct actions on smooth muscle (Bennett, Tyler, and Zaimis, 1957). Paralytic ileus has been observed in man (Grant and Boyd, 1957). In the hope of

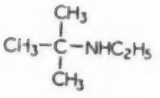
securing improvement in one or both of these respects we prepared and studied the ganglion-blocking actions of a variety of secondary and tertiary bases in which the nitrogen atom was sterically hindered by being closely surrounded by alkyl groups.



Mecamylamine



24605



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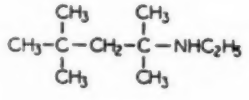

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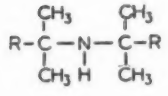
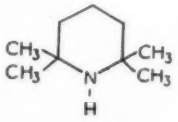


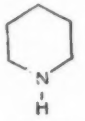
X



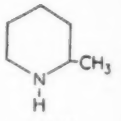
25636

Modest activity as a ganglion-blocking agent, about 10% of that of mecamylamine, was first seen in *NN*-dimethyl-*tert.*-octylamine (24605). A large number of similar tertiary alkylamines showed ganglion-blocking properties: the two best were *N*-ethyl-*tert.*-octylamine (25637) and *N*-ethyl-*tert.*-butylamine (25645), having activities of 20% and 30%, respectively, of that of mecamylamine.

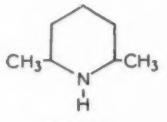
It was thought possible that increase in activity might be obtained by preparing substances in which the nitrogen atom was linked to two tertiary carbon atoms (**X**). The preparation of the simplest compound of this type, di*tert.*-butylamine (X, R = CH_3), is extremely difficult (Klages, Nober, Kircher, and Bock, 1941), and the yield is low (Brown, Barbaras, Berneis, Bonner, Johannesen, Grayson, and Nelson, 1953). Compounds containing two tertiary butyl groups are not only characterized by difficulty of formation but also possess relatively low stability (Brown and Barbaras, 1946). However, one cyclic analogue of di*tert.*-butylamine, 2:2:6:6-tetramethylpiperidine (25636), is readily accessible and is a very stable compound. We found it to be about twice as active as mecamylamine and to have other desirable properties, such as adequate persistence. We then prepared a wide variety of related heterocyclic compounds and have so far examined about 160. The results of this examination have supported our original theory that the essential feature of the molecule that confers activity is the close juxtaposition of several alkyl groups to a secondary or tertiary nitrogen atom. The influence of a number of alkyl groups in the 2- and 6-positions of the piperidine nucleus is well illustrated by the following series of secondary amines. Activities are shown as % of mecamylamine activity estimated on the nictitating membrane preparation.



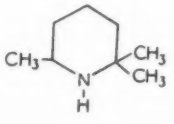
7387
Activity = 0



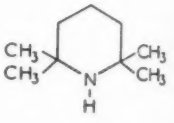
26539
Activity = 0



26388
Activity = 0



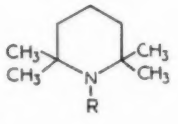
26725
Activity = 40



25636
Activity = 200

It is clear that activity increases as the number of methyl groups in the 2- and 6-positions increases. Again, if the 6-methyl group in 2:2:6-trimethylpiperidine (26725) is replaced by a higher alkyl group, activity increases along the series methyl, ethyl, propyl, *iso*propyl.

Tertiary amines were also studied: a wide variety of groups in the 1-position conferred high activity. Some examples are given below:



Compound	*R.*	*Activity*
25636	H	200
25745 (pempidine)	CH_3	250
26539	C_2H_5	300
29867	*n*-C_3H_7	200
26857	*n*-C_4H_9	150
28921	$CH_2.CH{=}CH_2$	120
26687	$CH_2.C_6H_5$	15

In other series, for instance, those having additional substituents in the 3- or 4-positions, there has also been a tendency for the 1-methyl or 1-ethyl homologues to show the highest activity. Quaternization of the tertiary amines caused loss of persistence: some quaternary compounds were highly active, but their action was rapid in onset and transient.

It is clear from the limited number of compounds shown that very many polyalkylpiperidines are highly active ganglion-blocking agents. Out of the total of 160 about 50 are as active as hexamethonium, the activity of which is equivalent to about 70 on the scale used, and about 20 are at least three times as active as hexamethonium. These potency ratios are of course likely to apply only in the particular conditions we used.

The examination of such a large number of highly active compounds was a formidable task. Our method was to select the ten compounds that, in the nictitating membrane test, had the highest potency coupled with a persistent action. We then examined each of these compounds by a second test: its effect on the responses of cat nictitating membrane, blood pressure and bladder to serial doses of 1:1-dimethyl-4-phenylpiperazinium (see Chen, Portman, and Wickel, 1954). Our object was to choose any compound that seemed to have specific anti-sympathomimetic actions, particularly on blood pressure. We observed no absolute specificity of this kind, but the results of these tests, and of quantitative studies of toxicity, showed that the most promising drugs of the whole group were pempidine and 26539, the 1-methyl and 1-ethyl derivatives of the first piperidine tested, 2:2:6:6-tetramethylpiperidine. The remainder of this report is concerned with the detailed study of these two compounds.

Methods

Compounds Used.—Some experiments were carried out with a sample of Inversine (mecamylamine hydrochloride) which was kindly given to us by Dr. H. Molitor. Most, however, were made with a sample of mecamylamine hydrochloride made by ourselves which proved identical with the sample provided by Dr. Molitor by infra-red analysis, and determination of melting point, mixed melting point, and C, H, and N contents. Ecolid (chlorisondamine chloride) and Ansolysen (pentolinium hydrogen tartrate) were kindly provided by Dr. H. J. Bein and Dr. R. Wien respectively. Hexamethonium bromide and tetraethylammonium bromide were made in our laboratories. Pempidine and 26539 were used either as hydrochlorides or hydrogen tartrates; the hydrochloride of the former was somewhat hygroscopic; that of the latter was very hygroscopic and was prepared in solution as required from the base. The physical properties of the bases and salts of pempidine and 26539 are given in Table I. Throughout this paper doses and concentrations of non-quaternary compounds refer to free base, and those of quaternary compounds to cation, unless otherwise stated.

Table I
PHYSICAL PROPERTIES

Salt	Pempidine	26539
Base	B.p. 184–186°. Slightly soluble in water. Miscible with common organic solvents	B.p. 196–199°. Slightly soluble in water. Miscible with common organic solvents
Picrate	M.p. 276° (dec.)	M.p. 245–247° (dec.)
Hydrochloride	M.p. 249° (dec.)	Hygroscopic
Hydriodide ..	M.p. 294–295° (dec.). Moderately soluble in water	M.p. 269–279° (dec.). Moderately soluble in water
Hydrogen tartrate	M.p. 160–160·2°. Very soluble in water	M.p. 158·5–160°. Very soluble in water
Hydrogen succinate	M.p. 118–120°. Very soluble in water	M.p. 118–120°. Very soluble in water

Nictitating Membrane.—Adult cats of either sex were anaesthetized by injection of chloralose, 80 mg./kg., into the saphenous vein. If this amount was inadequate it was supplemented with a second injection of 5 to 15 mg./kg. The right femoral vein and left femoral artery were cannulated for injection and blood pressure recording purposes, respectively, and a tracheal cannula was inserted. The right preganglionic cervical sympathetic nerve was severed and the rostral stump stimulated with rectangular pulses of approximately 8 V. and of 2.5 msec. duration, at a frequency of 4 to 5 sec. The contraction of the right nictitating membrane was recorded continuously, and drugs were injected intravenously during the sustained contraction. After the maximal effect of a dose had been observed, the nerve and membrane were usually rested for a time depending on the known or expected persistence of the drug used.

Potency Determination on Nictitating Membrane.—The high persistence of 26539, pempidine, and mecamylamine made determination of their potency difficult and time-consuming. It was found that a single intravenous injection of as little as 0.1 mg./kg. of 26539 or of pempidine, or of 0.2 mg./kg. of mecamylamine, markedly increased the sensitivity of the preparation for several hours. Two techniques were used. In the first a fresh cat was used for each dose of each of the compounds. In the second, a dose x, expected to be ineffective, or only slightly effective, was given (0.025 mg./kg. of pempidine or of 26539; 0.05 mg./kg. of mecamylamine), and when sufficient time had passed for the maximal effect to appear (usually 5 to 10 min.) another dose x was given, and followed in its turn by $2x$ and $4x$ in that order. It was assumed

that the successive cumulative effects produced by the four doses were those of x, $2x$, $4x$, and $8x$. This assumption seemed valid in view of the known persistence of the compounds and was supported by the finding that the results thus obtained did not differ significantly from those of the first technique. Whichever technique was used injections of one of two standard drugs, tetraethylammonium or hexamethonium, were first given to the same cat. When tetraethylammonium was to be studied three doses, 0.5, 1.0, and 2.0 mg./kg. (expressed as bromide), were given in random order. When hexamethonium was to be studied it was necessary to avoid delay and error due to persistence of large effects. A single dose of 0.5 mg./kg. (expressed as bromide) was therefore given and followed by a second dose of 0.25 or 1.0 mg./kg., according to whether the relaxation caused by 0.5 mg./kg. was greater or less, respectively, than 50%. Slope of effect against log. dose was determined when possible for each compound in each cat. Since the mean slopes thus determined for the five compounds did not differ significantly from the overall mean slope (32% relaxation for a two-fold increase of dose) this mean slope was used for all experiments and the dose causing 50% relaxation (ID50) was estimated for each compound in each cat. The mean ID50 and 95% confidence limits were then calculated in each case. The main source of error was the variation between cats.

Site of Action on Nictitating Membrane Preparation.—Two techniques were used in cats anaesthetized with chloralose. First, electrodes were applied to the rostral preganglionic stump of the cervical sympathetic nerve and to the unsevered postganglionic nerve. The two nerves were then stimulated alternately through a rotor with rectangular pulses (4 V., 2.8 msec., 4/sec.). Each nerve was stimulated for 12 sec. every 2 min., so that the membrane gave a sharp response every 1 min. The responses to pre- and postganglionic stimulation were readily made equal by small adjustments of voltage, width or frequency (the values given are those of Fig 2). Pempidine hydrogen tartrate was injected intravenously and its effect was observed until it declined or until the unaffected response to postganglionic stimulation deteriorated. Second, bipolar silver electrodes were applied to the rostral stump of the severed preganglionic cervical sympathetic nerve and to the caudal stump of the severed postganglionic nerve. The former nerve was stimulated with single rectangular pulses (1.5 V., 50 μsec.), using a Fleming Radio stimulator provided with an additional RF coupled output stage isolated from earth; the latter was built by our colleague, Mr. G. B. Horsfall, to the design of Schmitt and Dubbert (1949) and was found to be very satisfactory. The postganglionic action potential was amplified by a high gain, high discrimination resistance-capacity-coupled amplifier designed and built by Mr. Horsfall (time constant 1.0 sec., h.f. loss 15% at 200 c./sec.) and displayed for photography on a Cossor 1049 oscilloscope (time base, 150 msec.). The effects of an intravenous injection of pempidine were observed until maximal.

Pressor Responses.—Mean blood pressure was recorded from the femoral artery in cats anaesthetized with chloralose. Pressor responses were induced by five methods: (1) intravenous injection of acetylcholine after atropine; (2) injection of dimethylphenylpiperazinium; (3) injection of noradrenaline or adrenaline; (4) occlusion of both carotid arteries; (5) efferent stimulation of the right splanchnic nerve with rectangular pulses (10 V., 3 msec., 10/sec.). In each type of experiment, transient responses of the desired intensity, usually 60 to 80 mm. rise of pressure, were induced at constant intervals (usually 5 min.) and the effects of intravenous injections of drugs observed. As in experiments on the nictitating membrane mecamylamine, pempidine, and 26539 all had very persistent effects, and it was rarely possible to study the effect of more than one dose of each in each experiment unless the divided dose method was used.

Potency Determination by Pressor Response to Dimethylphenylpiperazinium. — As in nictitating membrane experiments two methods were used. In the first, a single dose of a single persistent compound was given intravenously to each cat. In the second, blocking doses in a series of x, x, $2x$, $4x$, $8x$, $16x$, etc., were injected, and two dimethylphenylpiperazinium injections given between each two blocking doses. Each cumulative effect was recorded as the mean of the two appropriate responses, expressed as a % of the mean of three control responses at the beginning of the experiment. This description applies only to mecamylamine, pempidine, and 26539: before any of these was studied effects of three doses of hexamethonium or tetraethylammonium were determined, and allowed completely to disappear.

A third method of assay was tried in which doses x_1, x_2, x_3, x_4, x_5 of a single persistent drug were calculated so that each new dose increased the cumulative total then given to $\sqrt{2}$ times the cumulative total at the previous dose. Alternate doses were then substituted by approximately equivalent amounts of another persistent drug, thus: x_1, y_2, x_3, y_4, x_5, y_6. The cumulative effect at each dose was recorded and analysis of the results was attempted. The technique appeared practicable, but analysis was exceptionally difficult, and the results of potency determinations by this method are not included, though they appeared not to differ greatly from results by the other two methods.

Heart Rate.—Heart rate was recorded in cats anaesthetized with chloralose by causing the chopped QRS complex of the electrocardiogram (lead II or, better, a precordial lead) to trigger a pulse generating circuit. The pulses were fed to a capacitor the charge on which was leaked off through a milliammeter from which the heart rate could be read directly, or were amplified to operate a Thorp impulse counter for kymographic recording. Changes in heart rate were induced by stimulating the distal stump of the severed

right vagus (30 V., 3 msec., 7/sec.) and their effects observed on the meter or on kymograph record of the Thorp impulse counter, and also by recording the mean femoral arterial pressure.

Gastric Secretion.—Male rats, weighing 90 to 110 g., were starved for 24 hr. and anaesthetized with ether. The pylorus was ligated, and doses of drugs or saline given subcutaneously or intra-duodenally just before or just after ligation. The rats were allowed to recover from the anaesthesia, and 6 hr. later they were killed and the gastric juices collected. Volume was measured, and the total and free acid were determined by titration using Topfer's reagent as indicator.

Actions on Gastrointestinal Movement and Saline Diuresis in Mice.—Groups of six male albino mice, weighing 18 to 22 g., were selected at random from a single stock cage in which the mice had food and water available *ad libitum* up to the time of experiment. They were treated by stomach tube with isotonic saline or solutions of salts of chlorisondamine, hexamethonium, pentolinium, mecamylamine, pempidine, or 26539 in isotonic saline. The concentrations were adjusted so that the dose for a 20 g. mouse was contained in 0.5 ml. All mice therefore received the same volume of fluid of approximately constant tonicity. Each group of mice was placed on 6 mm. mesh in a circular metabolism cage of 13.5 cm. diameter, above a funnel and a disc, 5 cm. in diameter, perforated with 1.5 mm. holes: this disc trapped faeces but allowed urine to pass. Urine volumes were measured at 30 min. intervals and faeces were collected after 5 hr., allowed to dry at room temperature overnight and weighed. In each experiment, two control groups received saline and six received drugs. The experiments were usually repeated six times for doses which seemed likely to contribute to calculation of the ID50, the dose that reduced the weight of the faeces to half. The very large numbers of mice used are shown in Table III. The plot of mean log. faecal weight against log. dose was linear, and ID50 values and confidence limits were calculated in the usual manner. Effects on saline diuresis were analysed graphically.

Actions on Bladder Tension.—An indirect indication of actions on bladder tension may have been provided by the "antidiuretic" effects observed in mice when high doses of ganglion-blocking agents were given. This action was also observed in the cat anaesthetized with chloralose by cannulating the bladder, filling it with warm saline, adjusting the pressure of the latter until the bladder became quiescent and recording the pressure with a float recorder. Usually blood pressure and contraction of the right nictitating membrane were also recorded. Responses of the three types were induced by serial injections of dimethylphenylpiperazinium as described for the dog by Chen *et al.* (1954). Between two such injections, a dose of ganglion-blocking agent was given, and injections of dimethylphenylpiperazinium continued until all responses returned to normal or until the preparation appeared obviously to have deteriorated. Often the latter occurred first. It was not possible to obtain satisfactory records of all three responses in all cats, the bladder response being the least reliable.

Antagonism in vitro.—Responses of the guinea-pig ileum to dimethylphenylpiperazinium, histamine, acetylcholine, and 5-hydroxytryptamine, and of the rat seminal vesicle to noradrenaline and adrenaline were established in the usual manner, and their antagonism by ganglion-blocking agents was studied. Effects of ganglion-blocking agents on spontaneous movements of rabbit duodenum were also observed.

Actions on the Neuromuscular Junction.—Twitch responses of the gastrocnemius muscle to electrical stimulation of sciatic nerve were observed in cats anaesthetized with chloralose. *In vitro* actions were studied on the rat phrenic nerve-diaphragm preparation of Bülbring (1946).

Cause of Death.—The lethal actions of large doses of pempidine were studied during recording of respiration with a Gaddum recorder, blood pressure, and electrocardiogram (lead II) of cats anaesthetized with chloralose.

Cardiotoxicity.—Effects of drugs were observed on the rate, amplitude of beat, and coronary flow (with a Thorp impulse counter) of the rabbit heart perfused with Ringer-Locke solution.

Central Nervous System Actions.—Central nervous system actions have been studied by five methods: (1) Observation in mice, rats, and dogs. (2) Measurement of the convulsant threshold. Convulsions were elicited in rats by application through ear electrodes of a 50 c./sec. alternating current of 7.5 mA and approximately 20 V. r.m.s.; the time taken for this current to elicit a maximal extension of the hind limb was measured and used to calculate the threshold in milliwatt seconds before and after treatment (Bogue and Carrington, 1953). Groups of ten rats (5 male and 5 female) were used, the individual animals being allocated before treatment so that all groups had the same mean threshold. (3) Antagonism to nicotine convulsions (see Stone, Mecklenburg, and Torchiana, 1956) was studied by giving drugs intraperitoneally 30 min. before intravenous injection of nicotine hydrogen tartrate (0.85 mg. base/kg.). (4) The sleeping times of groups of five mice previously treated with ganglion-blocking agents orally, and then given 100 mg./kg. of hexobarbitone intraperitoneally 2 hr. later, were compared with those of controls. The mice were maintained at a constant temperature of 34 to 36° by immersing their metal cages in a bath through which water was circulated by pump from a thermostat. Sleeping time was measured in each mouse as the duration of disappearance of the righting reflex. (5) Since tremor was the main central nervous system action observed, a method of measuring its intensity was designed. Groups of three mice received ganglion-blocking drugs or saline orally. At intervals of 20, 30, 40, and 60 min., and 1½, 2, and 2½ hr. the intensity of tremor in each group was

assessed by an observer, who did not know what drug any group had received and allotted a score from the following scale: violent tremor, 5; excessive, 4; medium, 3; slight, 2; very slight, 1. The total score for the seven observation periods was recorded as a measure of the liability of the drug to cause tremor. Every experiment but the first was carried out at a constant environmental temperature of 34 to 36°, to avoid possibility of error caused by hypothermia and shivering. The results were not obviously changed by warmth, and all have been analysed jointly.

Local Anaesthetic Tests.—Four methods were used. (1) Inhibition of the corneal reflex of rabbits by topical application to the eye. (2) The tail-root method of Bianchi (1956). (3) The guinea-pig weal method of Bülbring and Wajda (1945). (4) By recording the compound action potential of isolated frog sciatic nerve before and after application of solutions between the stimulating and recording electrodes.

Acute Toxicity.—Acute toxicity was determined in male albino mice weighing 19 to 21 g. Solutions of the salts were given by stomach tube (" oral " administration) or by injection, the dose for a 20 g. mouse being contained in 0.5 ml., except for an intravenous injection when this dose was contained in 0.2 ml. and given in 20 sec.

Chronic Toxicity.—Chronic toxicity has been studied in rats of both sexes by administration in three ways. (1) Solutions of hydrochlorides by stomach tube. (2) Solutions of hydrogen tartrates of pempidine and 26539, and of mecamylamine hydrochloride in the drinking water. (3) Mixtures of the two hydrogen tartrates or mecamylamine hydrochloride in the diet. Rats were weighed at least twice weekly and food intakes were measured at intervals. At autopsy, heart blood was withdrawn to estimate the haemoglobin concentration by the method of Sahli and serum cholesterol, in view of possible actions which the drugs might have on the coronary vessels. The following tissues were withdrawn, fixed in Zenker-acetic, sectioned and stained with haematoxylin-eosin: kidneys, adrenals, stomach, small intestine, large intestine, mesenteric lymph node, bladder, gonads, pancreas, spleen, liver, thymus, lungs, heart, thyroid, and pituitary.

Chronic toxicity has also been studied in male and female beagle hounds bred in our laboratories. Hydrogen tartrates of pempidine and 26539, and mecamylamine hydrochloride were prepared as scored, uncoated tablets containing 50 or 100 mg. These were shown to have short breakdown times. The dose for each dog was adjusted to the nearest half tablet, and given by hand once daily. Dogs were weighed weekly, and haematological studies were performed fortnightly. Dogs were observed carefully for toxic signs, which were sometimes recorded by colour cinematography on 16 mm. film. Occasionally it was necessary to treat eye infections (due to inhibition of lachrymation) topically with penicillin cream; no other drugs were given. At the end of each experiment dogs were killed by an injection of thialbarbitone intravenously and the tissues named above were taken for histological examination, and, in addition, the whole brain, the cervical cord, and the eyes. All but the last three were fixed in Zenker-acetic. Brain and cord were fixed in formol-saline and eyes in Szent-Györgi solution.

RESULTS

Actions on Nictitating Membrane, Preganglionic Stimulation.—The effects of mecamylamine, pempidine, and 26539 were qualitatively similar. Fig. 1 shows typical responses with 26539. Intravenous doses of 0.1 to 0.2 mg./kg. of pempidine or 26539, or of 0.2 to 0.4 mg./kg. of mecamylamine, caused a large fall of blood pressure (when the initial pressure was high), and a large relaxation of the nictitating membrane. These effects were slow in onset and persistent. In Fig. 1, the relaxation of the membrane with 26539 was maximal after 15 to 20 min. The retraction of the membrane was still only 65% of normal after 95 min. and about 85% of normal after 125 min., and had apparently returned to normal after 160 min. Even when the membrane was again contracting normally, it was possible to show an increased sensitivity to a further dose of the same or another ganglion-blocking agent, particularly when the original dose had produced an effect greater than 50%. Determination of potencies of any of the three agents was therefore very difficult. Even matching of very small responses to obtain approximate potencies appeared highly unreliable, and the values given above for compounds other than pempidine, mecamylamine, and 26539 were obtained by first estimating the likely range of potency in an experiment in which several compounds were injected in infrequent, just active doses, and then, in another cat, matching the maximal response of a single dose of one compound to one of the responses to several different doses of a compound having a transient action (such as tetraethylammonium) given earlier in the experiment. Such tests on interesting compounds were repeated as often as was necessary to give approximate potencies.

Accurate potency determination required many cats for each compound and the use of either the single dose, or the divided dose, technique described above. Analysis of the combined results of these two techniques gave the following values of ID50, the dose causing 50% relaxation, with 95% confidence limits, in mg./kg.: mecamylamine, 0.22 (0.16 to 0.31; 8 cats); pempidine, 0.092 (0.069 to 0.12; 11 cats); 26539, 0.072 (0.053 to 0.097; 10 cats); tetraethylammonium, 0.56

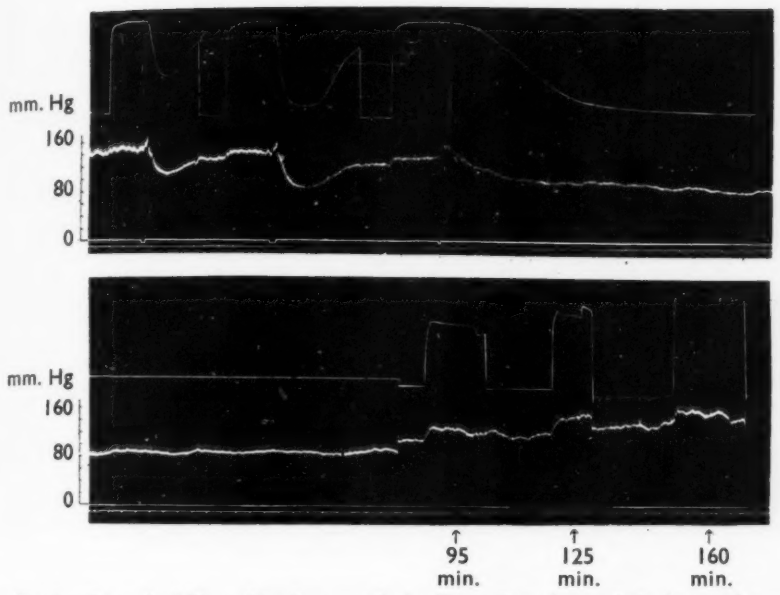


FIG. 1.—Cat, male, 4·6 kg., chloralose anaesthesia. From above down: nictitating membrane response to preganglionic stimulation; blood pressure; zero blood pressure and injection signal; time, min. Effects of tetraethylammonium, 1·0 (first signal) and 2·0 mg./kg. (second signal), and of 26539 (third signal), 0·2 mg./kg.

(0.43 to 0.71 ; 13 cats) ; hexamethonium, 0.30 (0.21 to 0.42 ; 8 cats). The five compounds were administered as hydrochloride, hydrogen tartrate, hydrogen tartrate, bromide and bromide, respectively. Here and subsequently all doses and concentrations are expressed as mg. base or cation.

These estimates of potency agree moderately well with those of Stone, Torchiana, Navarro, and Beyer (1956) and of Lee *et al.* (1958), when the likely differences in conditions of measurement in three different laboratories are taken into account.

Sites of Action on Nictitating Membrane.—Fig. 2 shows the results of a typical experiment in which pempidine was administered intravenously during repetitive brief responses of the membrane to alternating pre- and postganglionic stimulation. Although the amount of pempidine used, 0.5 mg./kg., was high in comparison with that causing a 50% relaxation during continuous stimulation, the response to postganglionic stimulation remained completely unimpaired ; that to preganglionic stimulation was much reduced. The baseline dropped slightly, perhaps indicating a continuance of ganglionic action throughout the experiment before pempidine was given. This might be attributed to the rather wide rectangular pulse used.

Fig. 3 shows the complete obliteration of the action potential in postganglionic nerve caused, in another experiment, by the intravenous injection of 0.5 mg. of pempidine/kg. The time course of this obliteration coincided approximately with that of the nictitating membrane response. The two records taken at 5 (*b*) and 10 min. (*c*) show an apparent increase in delay at the ganglion.

These two experiments demonstrate that pempidine acts at the ganglion, though its mode of action there requires study. The interesting

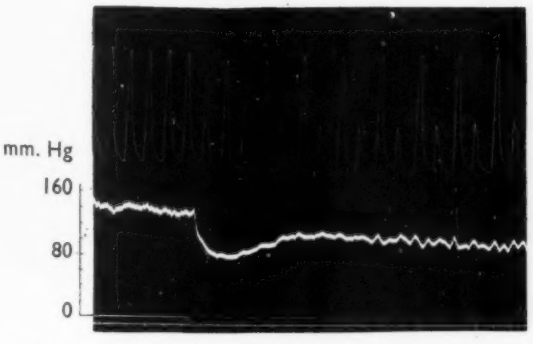


FIG. 2.—Cat, male, 3·2 kg., chloralose. From above down: responses of nictitating membrane to alternate pre- and post-ganglionic stimulation; blood pressure; zero blood pressure and injection signal; time, min. At signal, pempidine, 0·5 mg./kg.

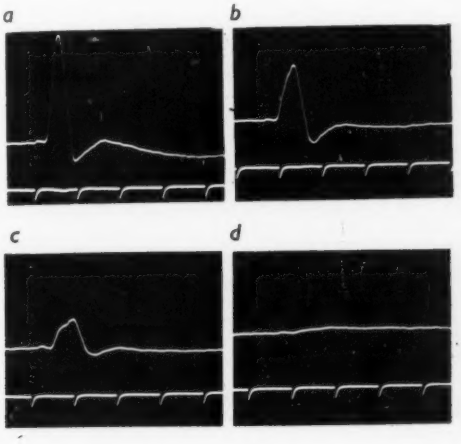


FIG. 3.—Cat, male, 2·6 kg., chloralose. Postganglionic action potentia l elicited by supramaximal preganglionic stimulation of the cervical nerve; time trace, 20 msec. (*a*) before; (*b*) 5 min., (*c*) 10 min., (*d*) 20 min. after pempidine, 0·5 mg./kg., given intravenously.

suggestion of Bennett *et al.* (1957) concerning the type of action of mecamylamine at the ganglionic synapse will be discussed later.

Actions on Blood Pressure.—Pempidine and 26539 caused a fall of blood pressure, the time course of which was closely similar to that of the action on the nictitating membrane (Fig. 1). Experiments studying the duration of action in various species have been carried out and particular attention has been given to: (1) measurement of the blood pressure of conscious normotensive rabbits, by cannulation of the central artery of the ear and capacitance manometry; (2) measurement of the blood pressure of conscious normotensive dogs, by passing an occluding cuff round the thigh and a detector cuff round the foreleg; (3) measurement in conscious normotensive and hypertensive rats by a cuff and photoelectric method; (4) measurement by cannulation in dogs anaesthetized with pentobarbitone.

The hypotensive actions recorded under all conditions were extremely variable. In general, pempidine 26539 and mecamylamine had most hypotensive action when the blood pressure was high. When the mean pressure was below 70 to 80 mm. the effect was often very small or absent. This prompted study of the effects of the three drugs on pressor responses to dimethylphenylpiperazinium, acetylcholine, carotid occlusion and efferent splanchnic stimulation. In some dimethylphenylpiperazinium experiments responses of nictitating membrane and bladder were also recorded.

Effects on Responses of Blood Pressure, Nictitating Membrane and Bladder to Dimethylphenylpiperazinium.—This test was used as one of the preliminary screening tests. % Inhibitions of response of blood pressure, nictitating membrane, and bladder, respectively, caused by the three drugs (each given as hydrochloride intravenously) were as follows: mecamylamine (0.1 mg./kg.; 2 cats) 63, 85, 44; 26539 (0.1 mg./kg.; 6 cats) 72, 100, 60; pempidine (0.05 mg./kg.; 2 cats) 61, 82, 59.

The nictitating membrane response was the most sensitive to all three drugs, and this finding was confirmed for each of the eleven drugs examined by this technique. Mecamylamine, pempidine and 26539 had similar potencies on bladder and blood pressure, but some other piperidines were found to have less hypotensive action and were discarded. Others were discarded because they were less active, or had brief or more toxic actions.

Repetitive pressor responses to dimethylphenylpiperazinium seemed to us to be more easily reproducible than those to nicotine or to acetylcholine. We did not observe tachyphylaxis when 10 to 30 μg. of dimethylphenylpiperazinium was given at intervals of 5 min. With nicotine tachyphylaxis was prominent, and with acetylcholine the response changed considerably during long experiments in control cats, probably because of declining atropine concentration. We therefore determined the potencies of five ganglion blocking agents as antagonists of the pressor response to dimethylphenylpiperazinium, using for mecamylamine, 26539 and pempidine both one dose/cat and divided dose methods, and for hexamethonium and tetraethylammonium the usual multi-dose methods. The divided dose method using pempidine is illustrated in Fig. 4. Pempidine and the other drugs inhibited the variable depressor (presumably the cardiac) response to dimethylphenylpiperazinium, as well as the pressor response. No attempt was made to measure the former inhibition. The results with both methods used for the three persistent drugs were analysed jointly. The following mean estimates of the ID50 with 95% confidence limits (mg./kg.) were obtained: mecamylamine, 0.081 (0.055 to 0.120; 9 cats); pempidine, 0.035 (0.023

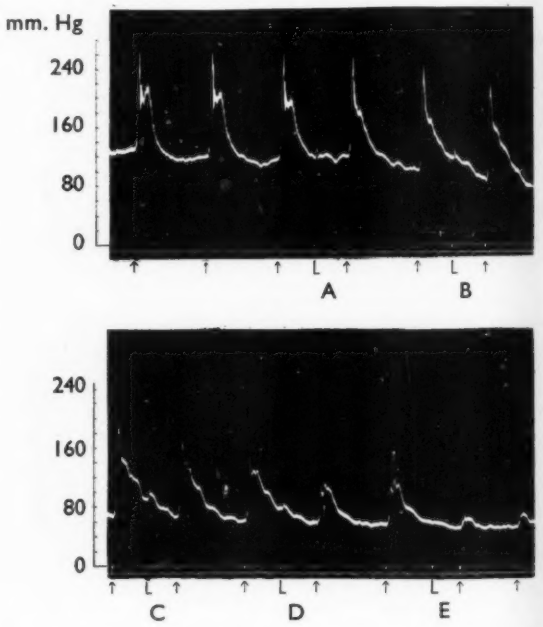


FIG. 4.—Cat, female, 2·5 kg., chloralose. From above down: blood pressure; zero blood pressure and injection signal; time, min. At arrows dimethylphenylpiperazinium, 10 μg./kg. At A, pempidine, 10 μg./kg.; B, 10 μg./kg.; C, 20 μg./kg.; D, 40 μg./kg.; E, 80 μg./kg.

to 0.055 ; 9 cats) ; 26539, 0.059 (0.042 to 0.083 ; 13 cats) ; hexamethonium, 0.037 (0.018 to 0.063 ; 4 cats) ; tetraethylammonium, 0.156 (0.093 to 0.262 ; 4 cats). The salts were those used in determinations of potency on nictitating membrane responses.

These results obtained with pempidine, 26539 and mecamylamine agree quite well with those obtained on the nictitating membrane, when allowance is made for the fact that the ID50 of pempidine was not significantly different from that of 26539 in either test. It seems possible that hexamethonium was more active than mecamylamine in the dimethylphenylpiperazinium test, and less active than mecamylamine in the nictitating membrane test. The number of cats used for the dimethylphenylpiperazinium test with hexamethonium was insufficient to justify great confidence in this finding, but we have certainly found no evidence for the opinion of Bennett *et al.* (1957) that the effect of mecamylamine on blood pressure is greater than would be expected from a comparison of its ganglion-blocking action with that of hexamethonium. However, they suggested that mecamylamine might have a central hypotensive action ; such an action would not have been observed under our experimental conditions.

Effects on Pressor Responses to Acetylcholine, Carotid Occlusion, Efferent Splanchnic Stimulation, and Noradrenaline.—Fig. 5 shows the effect of pempidine (0.1 mg./kg.) on the pressor response to acetylcholine in the atropinized cat, Fig. 6 that of pempidine (0.2 mg./kg.) on the response to carotid occlusion, and Fig. 7 that of 26539 (0.2 mg./kg.) on the response to efferent splanchnic

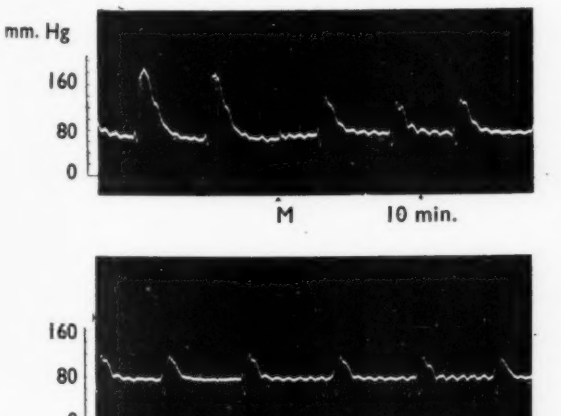


FIG. 5.—Cat, male, 2·8 kg., chloralose. Atropine sulphate, 4 mg. total. Records as Fig. 4. Effect of pempidine (M), 0·1 mg./kg., on responses to acetylcholine, 0·4 mg., at each signal.

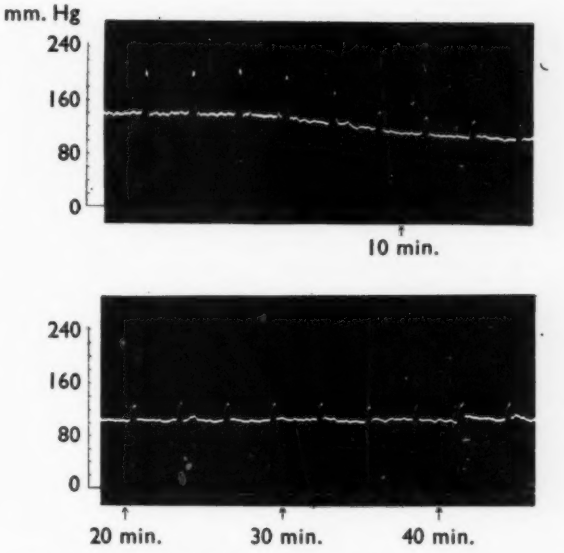


FIG. 6.—Cat, male, 2·7 kg., chloralose. Response of blood pressure to bilateral carotid occlusion (30 sec.). Records as Fig. 4. At signal, effect of pempidine, 0·2 mg./kg.

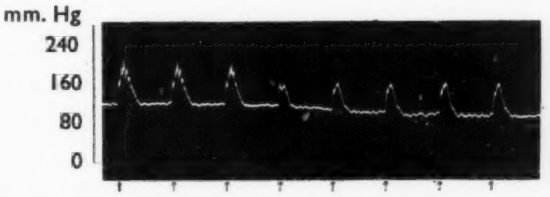


FIG. 7.—Cat, female, 2·7 kg., chloralose. Records as Fig. 4. At arrows, response of blood pressure to efferent stimulation of the right splanchnic nerve for 30 sec. At signal, effect of 26539, 0·2 mg./kg. The brisk vasoconstrictor response was unimpaired by 26539.

stimulation. Our results suggested that doses of 0.1 to 0.2 mg. of pempidine or of 26539 or of 0.2 to 0.4 mg. of mecamylamine/kg. markedly antagonized pressor responses to carotid occlusion and acetylcholine. The duration of the antagonism with each drug seemed similar to that observed in experiments on the nictitating membrane or on responses to dimethylphenylpiperazinium. The brisk response to splanchnic stimulation (partly mediated by postganglionic fibres) was very much less affected than was any other pressor response, and, upon evidence derived from experiments with four cats given pempidine and 26539, we considered that the slow response was probably somewhat less affected than responses to dimethylphenylpiperazinium, acetylcholine or carotid occlusion. Further experiments are required to clarify this problem.

Fig. 8 shows that mecamylamine and 26539, each given in high dosage (5 mg./kg.), failed com-

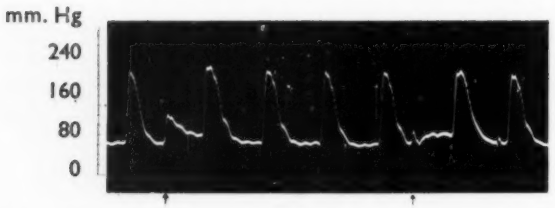


FIG. 8.—Cat, chloralose. Records as Fig. 4. Response of blood pressure to serial doses of noradrenaline, 2 μg., at signal. Effects of mecamylamine (first arrow), 5 mg./kg., and 26539 (second arrow), 5 mg./kg.

pletely to affect the pressor response to noradrenaline. In another experiment pempidine also had no effect.

These experiments support the view that the peripheral actions, at least, of the three compounds occur at the ganglion. Our experiments neither demonstrate nor disprove the existence of a central hypotensive action, but we are inclined to think that a powerful action of this kind is unlikely because of the similarity of effects on the carotid occlusion reflex to those on responses to hypertensive drugs.

Heart Rate.—Fig. 9 shows typical effects of pempidine (0.2 mg./kg.) given intravenously on the resting heart rate, and on the bradycardia and hypotension elicited by efferent vagal stimulation. In other experiments, mecamylamine (0.4 mg./kg.) by the same route had similar effects. The resting heart rate fell; the bradycardia and hypotension elicited by vagal stimulation were completely blocked. The latter action could be attributed to block of parasympathetic ganglion cells in the heart; the cause of the fall of the resting heart rate is less certain. A ganglion-blocking drug would be expected to cause bradycardia when control of the pacemaker is predominantly sympathetic, and tachycardia when control is predominantly parasympathetic, and it can be assumed that the bradycardia invariably observed in the cat is partly caused by block of the stellate ganglia, though we have not demonstrated this directly. Doubt whether this is a complete explanation arises from the observation (discussed below) that pempidine, mecamylamine and 26539 all cause a fall in rate of beat of the isolated, perfused rabbit heart. This directly-caused bradycardia may possibly contribute to that observed in the anaesthetized cat, though the concentrations that elicit it *in vitro* are high.

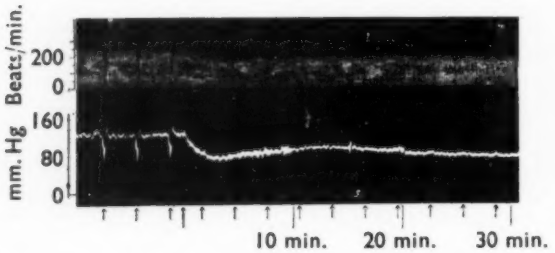


FIG. 9.—Cat, male, 2·6 kg., chloralose. From above down: heart rate (beats/min.); blood pressure record; zero blood pressure and signal; time, min. At small arrows efferent vagal stimulation (20 sec.); at large arrow, pempidine, 0·2 mg./kg.

Effects on Gastric Secretion.—Mecamylamine, pempidine and 26539 as hydrochlorides powerfully inhibited spontaneous gastric secretion of the rat. The results are given in Table II. When the effects of the subcutaneous injection of the drugs on the volume of secretion are compared the two piperidines appear between one and a half and two and a half times as active as mecamylamine. Doses which had effects by intraduodenal administration were only slightly higher than those active by subcutaneous administration, suggesting that oral absorption should be excellent (Lee *et al.*, 1958). The tartrates of pempidine and 26539 usually appeared to be less effective than the hydrochlorides, by either route; pempidine given intraduodenally, however, seems to be an exception.

TABLE II

DOSES OF GANGLION BLOCKING AGENTS THAT HALVE THE VOLUME, OR FREE ACIDITY OF THE SPONTANEOUS GASTRIC SECRETION OF THE RAT

s.c. = subcutaneous; i.d. = intraduodenal. 95% confidence limits may be calculated by multiplying and dividing the mean by 1·3 (vol.) or 1·5 (acid).

Comp.	Route	No. of Rats	ID50 (mg./kg.)	
			Vol.	Free Acid Conc.
Mecamylamine hydrochloride	s.c.	16	4·9	19
	i.d.	12	9·7	22
Pempidine hydrochloride	s.c.	12	2·9	12
	i.d.	12	10	22
Pempidine hydrogen tartrate	s.c.	22	4·8	15
	i.d.	12	7·6	23
26539 hydrochloride	s.c.	22	1·9	—
26539 hydrogen tartrate	s.c.	12	2·8	6
	i.d.	12	3·7	12

The three drugs also reduced the concentration of free acid, though the doses required were between two and a half and four times those that reduced the volume. Doses that reduced the concentration of free acid usually raised the concentration of total acid by 15 to 20%.

Effects on Excretion of Faeces by Mice.—Weights of faeces excreted by mice during the 5-hr. observation period varied widely, though all mice in a single experiment had a common food

and water supply during the preceding 24 hr. It was necessary to use over one thousand mice for the series of tests on six compounds. The results are shown in Table III. In spite of the large numbers used, the accuracy of each estimate of ID50 was low.

TABLE III

EFFECTS OF COMPOUNDS ON FAECAL WEIGHTS

ID50 is the dose reducing weight of faeces excreted in five hours to half and is recorded with 95% confidence limits.

Expt. Series	Comp.	No. of Mice	ID50
1	Mecamylamine hydrochloride ..	336	1·4 (0·9–2·3)
	Chlorisondamine chloride ..	180	10 (5·8–17)
	Pentolinium bromide	156	6·5 (4·7–10)
	Hexamethonium bromide ..	96	40 (32–49)
	Pempidine hydrochloride ..	354	1·5 (0·9–2·3)
2	Mecamylamine hydrochloride ..	72	0·8 (0·5–1·3)
	26539 hydrogen tartrate.. ..	72	0·4 (0·2–0·9)

Small oral doses of mecamylamine, pempidine, and 26539 reduced gastrointestinal movement as judged by the weight of faeces excreted within 5 hr. All three drugs were much more active than chlorisondamine, pentolinium or hexamethonium. The low activity of these quaternary compounds given orally might be ascribed either to poor absorption and failure of unabsorbed compound to act directly, or to low intrinsic activity. Further information on this important point could probably be obtained by estimating ID50 by our method for a number of compounds given subcutaneously, intramuscularly or intravenously as well as orally. We consider on the basis of the present observations, and of values of oral and parenteral LD50, that the most probable reason for the high activity of pempidine and mecamylamine is their excellent absorption, rather than exceptionally high intrinsic potency on the gastrointestinal tract.

Effects on Saline Diuresis in the Mouse.—During each of the experiments on faecal excretion, urine volumes were measured at 30 min. intervals. The total excreted in 5 hr. has been used to compare effects of drugs. The main findings were as follows.

Mecamylamine had little effect on urine volume when doses smaller than about 8 mg./kg. were given. Higher doses had "antidiuretic" effects. The urine volume was halved by approximately 25 mg./kg. and very little urine was excreted after 40 mg./kg. Pempidine seemed to have less "antidiuretic" action and doses as high as 40 mg./kg. reduced the urine volume by only 30%. The ID50 calculated by extrapolation was about 80 mg./kg.

Chlorisondamine chloride had no effect in doses up to 9 mg./kg. Very little urine was excreted after doses of 45 mg./kg. The ID50 was about 25 mg./kg. Pentolinium hydrogen tartrate had no effect in doses up to 5 mg./kg. Very little urine was excreted after doses of 50 mg./kg. The ID50 was about 25 mg./kg. Hexamethonium had no effect in doses of 55 mg./kg.; nor had 26539 in doses of 5 mg./kg.

The cause of the reduction in urine volume has not been investigated, but is presumed to be difficulty of micturition. Pempidine and mecamylamine strongly inhibit the bladder contraction elicited by dimethylphenylpiperazinium and probably reflex contraction. It seems possible that mecamylamine and pempidine have some advantage over pentolinium and chlorisondamine in this respect, if allowance is made for the supposed poor absorption of the last two drugs when they are given orally.

Pempidine, though more potent than mecamylamine by most tests hitherto discussed, caused much less urinary retention.

Actions on Responses to Agonists in vitro.—Each result is expressed as the negative logarithm (pA_2) of the molar concentration of antagonist that reduced the effect of $2x$ μg. of agonist to that shown by x μg. of agonist in the absence of antagonist (Schild, 1947). The results are given in Table IV.

TABLE IV

ANTAGONISM OF VARIOUS AGONISTS BY GANGLION BLOCKING DRUGS

Adrenaline and noradrenaline were tested on the rat seminal vesicle preparation, other agonists on the guinea-pig ileum preparation. NE indicates no effect at stated molar concentration. The number of experiments is given in parentheses.

Agonist	pA_2		
	Mecamylamine	Pempidine	26539
Dimethylphenylpiperazinium	5·75 (3)	6·15 (4)	6·00 (9)
Acetylcholine	3·55 (5)	3·35 (3)	3·40 (4)
Noradrenaline	NE 3·30 (3)	NE 3·30 (3)	3·60 (2)
Adrenaline	NE 3·30 (3)	NE 3·30 (3)	3·85 (4)
5-Hydroxytryptamine ..	NE 3·30 (2)	NE 3·30 (2)	NE 3·30 (2)
Histamine	3·60 (1)	NE 4·20 (1)	NE 4·20 (1)

All three compounds were powerful antagonists of dimethylphenylpiperazinium; mecamylamine was the least active, as in every other test of ganglion blocking activity used. All three were very feeble antagonists of acetylcholine; the concentrations recorded can have no relevance *in vivo*. Compound 26539 was a feeble antagonist of noradrenaline and adrenaline; mecamylamine and pempidine were not. No compound was an antagonist of 5-hydroxytryptamine. Mecamylamine was a very feeble antagonist of histamine.

Actions on Isolated Rabbit Duodenum.—Mecamylamine in a concentration of 40 mg./l. reduced the tone of spontaneously contracting rabbit duodenum, while 80 mg./l. reduced both tone and amplitude. Compound 26539 (80 mg./l.) had only small effects on tone.

Though these experiments might have been taken to suggest an advantage of piperidines over mecamylamine, we thought that the concentrations used would be unlikely to be achieved *in vivo*, and discontinued the experiments.

Actions at the Neuromuscular Junction.—Pempidine and 26539 had only very feeble blocking actions at the neuromuscular junction, being 100 times less potent than at the autonomic ganglion. Intravenous doses of 10 mg./kg. caused a protracted reduction in the twitch of cat gastrocnemius muscle elicited by sciatic stimulation by 20 to 30%. Doses of between 2.5 and 5 mg./kg. of either drug had little or no effect alone but seemed to potentiate the effect of tubocurarine and had some effect when given during the action of a dose of tubocurarine (Fig. 10). In an extensive study of the mode of action of mecamylamine at the neuromuscular junction, Bennett *et al.* (1957) obtained similar results (see also Stone, Torchiana, Navarro, and Beyer, 1956).

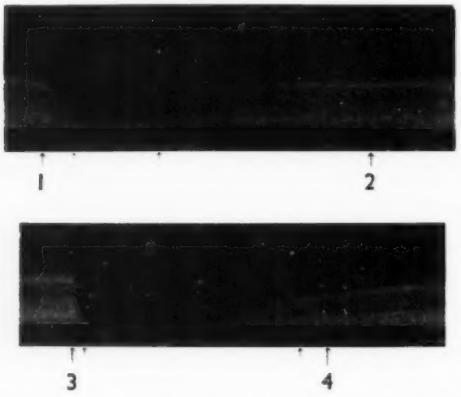


FIG. 10.—Cat, male, 3·9 kg., chloralose. From above down: twitch of gastrocnemius muscle elicited by stimulation of the sciatic nerve; injection signal; time, min. Injections at signals: (1) tubocurarine, 0·2 mg./kg.; (2) pempidine, 5 mg./kg.; (3) tubocurarine, 0·2 mg./kg.; (4) pempidine, 2·5 mg. kg. The two records were continuous. Artificial respiration was given between small arrows.

The actions of mecamylamine and 26539 on the rat phrenic nerve-diaphragm preparation of Bülbring (1946) were also studied. Mecamylamine (40 mg./l.) reduced the twitch height to 8% of its initial value within 6 min. Nine washes each lasting 30 min. were necessary to restore the twitches to their initial height. Compound 26539 had no effect at 40 mg./l., but at 80 mg./l. caused a very slow reduction of contraction, ending in complete block after 40 min. Though positive effects were obtained, the concentrations used were very large, and the experiments were therefore discontinued.

Cause of Death.—Large intravenous doses of 26539 and pempidine hydrogen tartrates were given to one and three anaesthetized cats, respectively. As their effects were qualitatively identical, only those of pempidine will be described. Serial doses of 10 mg./kg. were administered intravenously at intervals of 5 min. until death occurred (Figs. 11 and 12). In each of the three experiments irregular gasping respiration occurred after 70 to 80 mg./kg. had been injected and was succeeded by apnoea after about 100 mg./kg. had been injected in two cats to whom artificial respiration was not given (Fig. 11). The mean blood pressure fell after the first dose to about 100 mm. and no further fall occurred: indeed, the pressure tended to rise slowly. In those two cats in which apnoea was allowed to occur, there was a more pronounced increase in blood pressure during the last 10 to 15 min. of dyspnoea (Fig. 11). The circulation deteriorated rapidly after the respiration stopped. Before this the condition of the heart, as judged from the electrocardiogram, lead II, remained normal except for bradycardia, a

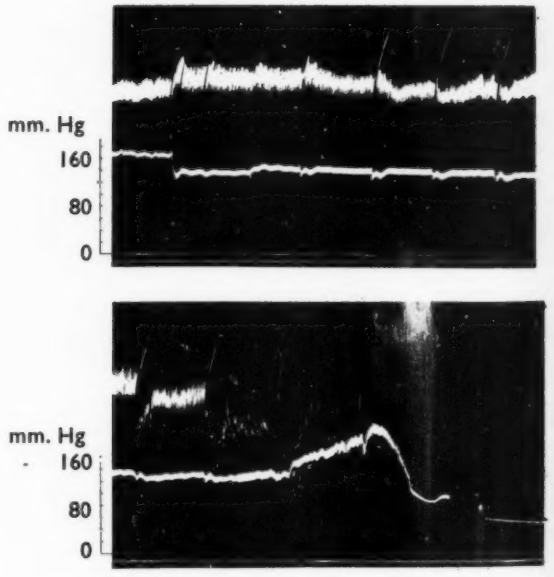


FIG. 11.—Cat, female, 3·2 kg., chloralose. From above down: respiration; blood pressure; zero blood pressure and injection signal; time, min. At signals 10 mg. pempidine/kg., intravenously. No artificial respiration.

lengthening of the PR interval and slight depression of the T wave. In another cat in which artificial respiration was given as soon as dyspnoea was marked, a large dose of pempidine (in all 120 mg./kg.) had no effect on heart or blood pressure other than that seen during the earlier stages of the experiment (Fig. 12). There was no late hypertensive phase. Artificial respiration was then stopped, and the cat died in the same manner as the other two.

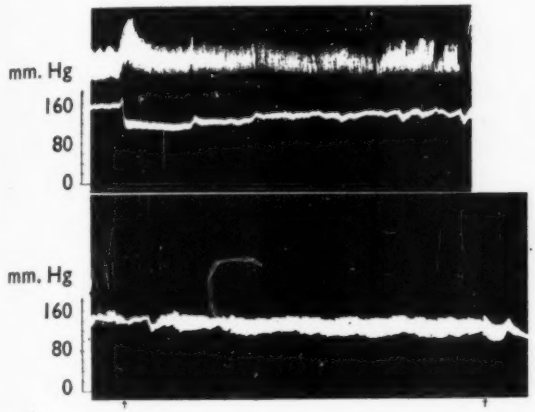


FIG. 12.—Cat, male, 3·5 kg., chloralose. Records as Fig. 11. At signals pempidine, 10 mg./kg., intravenously (total dose 120 mg./kg.). Artificial respiration was given between the arrows.

The cause of the respiratory changes is not known. Obvious possibilities are a central action, or an action at the neuromuscular junction. The experiments with the gastrocnemius muscle suggest that part, at least, of the respiratory paralysis may be due to neuromuscular block.

The cause of the late hypertensive phase is also unknown. As it only occurred in the two cats not given artificial respiration, it may be asphyxial in origin. Whatever its cause it presumably involves some activity of the sympathetic system, which must therefore be incompletely blocked in spite of a dose of pempidine one thousand times greater than the minimal doses causing a moderate hypotension. Payne and Rowe (1957) gave mecamylamine to cats in serial doses of 10 mg./kg., and observed transient but marked hypotensive responses to each new dose, although, as in our own experiments, the general level of pressure tended to rise slowly. We did not see these transient responses after pempidine and consider that, under these conditions, the actions of mecamylamine and pempidine are different.

Actions on Perfused Rabbit Hearts.—Bennett *et al.* (1957) showed that mecamylamine reduces the rate and amplitude of beat of the isolated hearts of cat, rabbit and guinea-pig. We have confirmed these observations on the rabbit heart and have also found that mecamylamine reduces the coronary flow (Fig. 13). Pempidine and 26539 have qualitatively similar actions, though pempidine is less active.

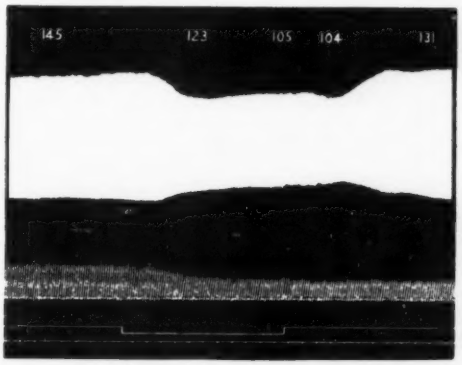


FIG. 13.—Isolated rabbit heart preparation perfused with Ringer-Locke solution. From above down: rate; amplitude; coronary flow; perfusion signal; time, min. During signal, heart perfused with mecamylamine, 20 mg./l.

Twelve isolated rabbit hearts were perfused with Ringer-Locke solution. A fresh preparation was used for each experiment with each concentration of each drug. During perfusions with 10 mg./l. the following maximal % effects were observed on amplitude, rate and coronary flow respectively: mecamylamine, −20, −30, −30 (2 hearts); 26539, −14, −16, −33 (4 hearts); pempidine, 0, −6, +10 (1 heart). At 20 mg./l. the effects were: mecamylamine, −54, −10, −26 (2 hearts, no rate determination in one); 26539, +14, −16, −46 (1 heart); pempidine, −19, −4, −8 (1 heart). The effects of pempidine at 40 mg./litre were: −37, −16, −20 (1 heart).

On the basis of these few observations, it seems that pempidine is about one quarter to one half as toxic as mecamylamine to the isolated heart and that compound 26539 is not much less toxic than mecamylamine.

Tremor and Other Central Actions.—Tremor is one of the most important clinical side effects of mecamylamine, though the reported incidence is not high. It is also interesting because it might be caused by interference with central transmission by acetylcholine. We have studied the comparative liability of mecamylamine, pempidine and 26539 to cause tremor and have been able to induce tremor with high doses of all three agents in mice, rats and dogs. We have done no more than record its occurrence in rats, but have carried out comparative experiments in mice and dogs.

The scoring method of assessing tremor in mice, when all tests were carried out by a single, highly skilled observer who never knew which drug had been given to any group, gave surprisingly consistent results. For instance in three experiments, each of which included three doses of each drug, the three doses in each experiment were always scored in the expected order and the means from the three experiments provided excellent plots of effect against log dose. It was often found easier to award scores if the mouse was placed in the palm of the hand, but care had to be taken to distinguish tremor from respiratory movements.

The highest possible hypothetical score was 35, which would have represented the occurrence at each time of observation of a continuous violent tremor. A score of 20 was selected for the calculation of active doses, and usually represented a protracted, readily perceptible but rather fine, rapid tremor. The amounts of the drugs (expressed as mg./kg. by mouth) calculated to give this score with their 95% confidence limits were: mecamylamine hydrochloride, 38 (30 to 48); pempidine hydrogen tartrate, 100 (79 to 126); 26539 hydrogen tartrate, 81 (64 to 102).

The higher toxicity of mecamylamine in this respect was confirmed by studies during chronic toxicity tests in beagle hounds, described in detail later. Two groups, each of two bitches and one male, received 26539 and pempidine, 25 mg./kg., and two males received mecamylamine, 25 mg./kg. Other males and bitches received 5 mg./kg. of pempidine or 26539 and at no time did these dogs exhibit tremor. Both dogs receiving mecamylamine showed tremor from about the fourth day onwards. In one (981), it had become severe by the fourth week and was of a very coarse, flapping type (see Perry and Schroeder, 1957) and was associated with a marked abnormality of stance and gait; though the drug was withdrawn on the fifth day of the fourth week, the dog became comatose and died two days later. The other dog receiving mecamylamine (980) showed moderate tremor throughout the eight weeks during which it received the drug. Although this tremor was inconstant, it could always be seen on occasions during any period of observation longer than a few minutes and often seemed to simulate what has been described clinically as intention tremor, since it almost invariably occurred just before the dog stood or moved. The tremor was of the moderately coarse but never the flapping type.

Neither of the bitches which received 25 mg./kg. of pempidine showed tremor at any time. On rare occasions a very mild tremor, barely perceptible, was observed in one of the two bitches which received 25 mg./kg. of 26539. The two dogs receiving 25 mg./kg. of 26539 or pempidine usually showed tremor during any short period of observation, but the tremor was much less marked than that seen in dog 980: episodes of tremor were invariably very mild and very brief. Cinematograph records were made of the signs in these two dogs and in the two dogs receiving mecamylamine.

We did not attempt to induce more pronounced tremor by giving larger doses of pempidine or 26539, since a preliminary experiment had shown that higher doses caused ocular discomfort and since our experiments seem to have established that pempidine and 26539 were both capable of inducing tremor but were much less active than mecamylamine in this important respect.

With the central effects of hyoscine and benactyzine in mind, it is of interest to enquire whether pempidine, 26539 and mecamylamine, which may interfere with the activity of central cholinergic neurones, might give rise to signs of central effects other than tremor, particularly in view of the clinical reports of the central actions of mecamylamine—for example, Perry and Schroeder (1957). We have not seen, however, striking behavioural changes in mice, rats or dogs receiving any of the three ganglion-blocking drugs. All dogs receiving these drugs, except 980 and 981, remained bright, alert and friendly throughout the chronic toxicity tests. In dog 981 listlessness and withdrawal were observed, but could be considered secondary to the motor disturbance and to the deterioration in general condition. The same signs were also seen to a lesser extent in dog 980.

Rats and mice displayed what might be described as stupor after single doses of 25 mg./kg. of pempidine or 10 mg./kg. of mecamylamine intraperitoneally. The animals were readily aroused, and might well be displaying no more than a response to the discomfort which such doses might be expected to cause. Oral doses of 25 mg./kg. of the three drugs had no effect on the mean sleeping time of mice given hexobarbitone (100 mg./kg. intraperitoneally) 2 hr. later. Benactyzine (5 mg./kg.) caused a large increase in sleeping time under these conditions.

All three drugs raised slightly the threshold to electrical convulsions when given to rats in doses of 10 to 50 mg./kg. Mecamylamine (50 mg./kg.) appeared to protect 3 rats out of 10, the protection being that defined by Bogue and Carrington (1953) in their description of this test. All three drugs also had a curious effect not previously

observed in these laboratories during studies of the action of several hundred potential anticonvulsant drugs. Consciousness was lost by rats during and for a short time after electrical convulsions and respiration was briefly depressed. Rats receiving non-lethal doses of 5 to 25 mg./kg. of 26539 or 5 to 50 mg./kg. of mecamylamine or pempidine often failed to breathe again after their convulsions. The approximate LD50 was 25, 17 and 25 mg./kg. for the three drugs. A possible theoretical explanation is that the cause of these deaths was respiratory depression, not lethal by itself but made so by the addition of post-convulsant depression.

We have confirmed the finding of Stone, Mecklenburg and Torchiana (1956) that mecamylamine prevented convulsions induced by nicotine, an action which we have also found with pempidine and 26539. Tremor was not prevented. Though 470 mice were used there were considerable differences between the ED50 values obtained in two different experiments. Pempidine and 26539 seemed to be somewhat more active than mecamylamine; hexamethonium was about one-fifth to one-tenth as active as mecamylamine. We are continuing other studies of the central actions of these drugs.

Local Anaesthetic Tests. — Mecamylamine hydrochloride, pempidine and 26539 (as hydrogen tartrates) were used for all local anaesthetic tests. All concentrations given are w/v.

1% solutions of the three compounds applied to the rabbit eye did not affect the corneal reflex. Stone, Torchiana, Navarro and Beyer (1956) obtained a similar result with 1% mecamylamine.

Injection of 0.05 ml. of a 2% solution of each of the three drugs subcutaneously into the tail root (Bianchi, 1956) had no local anaesthetic effect as judged by the aversive response of the mice to tail pinching and produced no toxic signs. Injection of the same volume of a 4% solution of each drug had apparent small, brief local anaesthetic effects, but this concentration caused severe prostration. We concluded that local anaesthetic action was not demonstrated by these experiments. Lignocaine 2% gave complete local anaesthesia with this method.

The method of Bülbring and Wajda (1945) gave evidence of local anaesthetic activity. Solutions of the three ganglion blocking agents or of procaine hydrochloride were injected intradermally into guinea-pigs in volumes of 0.25 ml. Four positions on each of 16 guinea-pigs were used and doses were allotted from a Latin square design. The reflex responses to a total of 48 pricks in each weal area, given in 8 blocks of 6 during 40 min., were halved by the following concentrations of bases (derived graphically): procaine, 0.15%; mecamylamine, 0.30%; pempidine, 0.35%; 26539, 0.45%. In some experiments tests were continued at intervals from 40 min. to 4 hr. All effects were reversible. That of procaine (0.25%) faded between 1 and 2 hr., that of mecamylamine (0.5%) between 40 min. and 1.5 hr., and those of 26539 and pempidine (0.5%) between 30 and 80 min.

Compound 26539 (0.25% or 2.5%) when applied to isolated frog sciatic nerve between stimulating and recording electrodes caused an increase of about 10% in the height of the action potential elicited by supramaximal stimulation. Mecamylamine (1 or 2.5%) caused a slow fall of action potential to about 80% of the control height after 10, 20% after 20, and 0% after 30 min.; 0.25% solutions had little effect. This change was irreversible though nerve was left to soak in fresh frog Ringer solution for as long as 2 hr. Consequently it is uncertain whether the action of mecamylamine is to be regarded as toxic or anaesthetic and further study is desirable.

The likeliest explanation of our results is that the three compounds potentially have local anaesthetic action but are unable readily to penetrate nerve trunks. In the Bülbring and Wajda (1945) test, the drugs are brought into intimate contact with nerve endings, with single fibres and with collections of a few fibres. Their powerful action in this test presumably reflected their ability to penetrate one, some, or all of these anatomical entities.

Acute Toxicity.—Results of acute toxicity determinations are given in Table V. Some of the values given earlier by Spinks and Young (1958) have been modified by additional experiments. The values for mecamylamine are nearly identical with those given by Stone, Torchiana, Navarro, and Beyer (1956), but those for pempidine are higher than the values recently published by Lee *et al.* (1958).

Mecamylamine was more toxic than 26539 and 26539 was more toxic than pempidine. The hydrogen tartrates and hydrochlorides were equally toxic. All compounds were well absorbed orally, as judged by the ratio of oral to intravenous LD50. In this respect, the results agree well with those on gastric secretion and with those of biochemical studies of Lee *et al.* (1958). However, the ratio of oral to intravenous toxicity was somewhat lower for mecamylamine than for

TABLE V

ACUTE TOXICITY OF COMPOUNDS

All doses expressed as base. The number of separate experiments contributing to each mean was 3 unless otherwise shown in parentheses after the number of mice. 95% confidence limits follow LD50 values in parentheses.

Comp.	Salt	Intravenous		Intraperitoneal		Oral	
		Total No. of Mice	LD50 (mg./kg.)	Total No. of Mice	LD50 (mg./kg.)	Total No. of Mice	LD50 (mg./kg.)
Pempidine	Hydrogen tartrate	57	64 (50–83)	55	93 (65–134)	110 (5)	412 (292–581)
	HCl	39	74 (57–96)	59	125 (87–179)	99 (5)	413 (293–582)
26539	Hydrogen tartrate	54	37 (28–48)	80	43 (30–62)	85 (5)	139 (89–218)
	HCl	53	43 (33–56)	56	48 (33–69)	81 (4)	148 (101–218)
Mecamylamine	HCl	45	21 (17–28)	54	37 (25–53)	84	96 (62–151)

pempidine, which may therefore be less rapidly absorbed or more rapidly eliminated.

Chronic Toxicity to Rats.—Preliminary tests in which pempidine, 26539, and mecamylamine were given by catheter once on each of 5 days a week for 2 weeks to groups of 6 rats, showed that the maximum tolerated daily dose of each was of the order of 50 mg./kg. This dose caused loss of weight (see Lee *et al.*, 1958) and condition, but did not kill any animals. Liver and kidney were unaffected by the drugs.

The most convenient method of administering soluble drugs for long periods is to dissolve them in the drinking water, and we next carried out a pilot test in which each of the drugs was so administered at a concentration of 0.025% to groups of 5 rats. The control water intake of 208 ml./kg. (giving an expected daily dose of 52 mg./kg.) fell immediately to 65 ml. in the mecamylamine group, 72 ml. in the pempidine group, and to 48 ml. in the 26539 group. After 8 days these intakes had risen to 190, 168, and 170 ml. respectively, but by this time in the respective groups 1, 2, and 3 rats had died. We thought that dehydration probably contributed to the cause of these deaths, and the experiment was abandoned.

The full scale test on rats was conducted in the following manner. Each group of 10 male and 10 female rats (90 to 100 g.) received drug in the diet. Three groups received 26539 in concentrations of 0.05, 0.015, and 0.005%, three received pempidine in the same concentrations, and two received mecamylamine in concentrations of 0.05 and 0.15%. Individual weights and the food intakes of each half group were recorded daily for the first 2 weeks. Thereafter, food intakes were recorded at weekly, and weights at 3 to 4 day intervals. Half the rats were killed and submitted to autopsy at 8 weeks; the remainder were maintained on the drugs for fertility and carcinogenic tests. Fertility tests were commenced at 17 weeks, experimental females in oestrus (as indicated by daily vaginal smears) being mated with normal males, and experimental males with normal females in oestrus. Females of all groups have given litters. The results of tests on males are incomplete. These experiments are still in progress. Body weights and food intakes become unreliable after 17 weeks and have not been reported. Deaths occurred only in the groups receiving 0.05% of drugs. In the 26539 group one male died on the 3rd day; a second developed paralytic ileus in the 11th week and was killed; a third died in the 18th week. One female died in the 7th week. In the pempidine group one male developed paralytic ileus in the 14th week and was killed. In the mecamylamine group one male was in very bad condition in the 9th week and was killed. This experimental induction of paralytic ileus is, as far as we are aware, the first to be reported. It is interesting that it was observed only after treatment for between three and four months with these relatively enormous doses of drugs, being about 50 times those causing considerable faecal retention in mice.

The food consumption of all experimental groups dropped sharply during the first few days of drug administration. The averages of the experimental groups for the first 3 days ranged between 53 and 72 g./kg. against 100 g./kg. for the control group. Thus, concentrations as low as 0.005% were readily detected by the rats and disliked. By the end of the first week intakes in g./kg. were again similar to those of the control groups and they remained similar up to the 17th week. This means that the animals that grew least ate least.

Control intakes fell steadily from between 100 g./kg. and 92 g./kg. in the 3rd week, to 82 in the 6th, 77 in the 9th, and 62 in the 14th week. Drug intakes therefore fell correspondingly, for example, from 50 mg./kg. at the beginning to about 30 mg./kg. in the 14th week in the groups receiving the highest doses. Generally, females ate about 10% less (g./kg.) than males in all

groups and therefore received slightly lower doses. The values given are means of the results in the two sexes.

The effects of the drugs on growth are shown in Table VI. The effects of mecamylamine were much the most severe; those of pempidine were very slight. We estimate that the concentrations in food causing a 15% reduction in weight gain at the 8th week were: mecamylamine, 0.0125%; 26539, 0.029%; pempidine, 0.06%. The plots of effect against log concentration were far from parallel and comparative toxicities in this respect would therefore be different if a different weight reduction were selected.

TABLE VI

GROWTH OF GROUPS OF 20 RATS RECEIVING DRUGS IN THE DIET

Values for males and females are averaged. Half the rats were killed after 8 weeks.

Drug	% Conc. in Food (w/w)	Mean Weights			Weight Gain as % of Control Weight Gain	
		Day 0	8 Weeks	17 Weeks	8 Weeks	17 Weeks
Control ..	—	94	200	218	(100)	(100)
Mecamylamine	0·05	102	157	187	52	69
	0·015	103	187	206	79	86
Pempidine ..	0·05	92	183	210	86	96
	0·015	94	192	225	92	103
	0·005	93	195	222	98	104
26539	0·05	92	176	180	79	71
	0·015	96	192	212	91	94
	0·005	96	213	234	110	112

Compound 26539 caused an apparent increase in growth at a concentration of 0.005%.

No macroscopic changes were observed at autopsies at the eighth week, and the mean blood cholesterol concentration and the mean haemoglobin concentration then were approximately the same in all groups.

Histological studies by our colleague Dr. Paget of the tissues named in the Method showed no changes attributable to mecamylamine (compare Stone, Torchiana, Navarro, and Beyer, 1956) or pempidine, but compound 26539 caused changes in pituitary and testis which are still under investigation and which have led us to defer clinical trial of this compound.

Chronic Toxicity to Dogs.—A preliminary experiment in which mecamylamine, 26539, or pempidine was each given to one beagle hound daily for 4 weeks showed that the highest tolerated dose was determined by the ocular rather than the lethal or gastrointestinal effects. We began by giving 25 mg./kg. daily as tablets. Though some abdominal distension and ineffective straining to defaecate were occasionally observed in each dog, these effects were never more than slight, and appetite appeared to be unimpaired. The ocular effects were pronounced. We observed a dilated pupil in strong light, a relaxation of the nictitating membrane, a block of lachrymal secretion, and signs of what we considered to be a difficulty of accommodation. Secondary to these effects blinking and a mild suppurative conjunctivitis developed, the latter readily controlled by topical treatment with penicillin cream. After 2 weeks it appeared that 25 mg./kg. caused no, or only trifling, discomfort. We then raised the dose to 50 mg./kg., but the ocular signs immediately worsened and began to cause discomfort. The dose was therefore reduced to 25 mg./kg. after 3 days. The only other severe effect seen was dryness of mouth and tongue.

The dogs were killed after 4 weeks and examined *post mortem*.

The large scale chronic test in dogs involved the daily administration, for 5 days a week, of 25 mg./kg. pempidine and 26539, each to two bitches and one male dog, and of 5 mg./kg. of each drug to one bitch and two male dogs. Mecamylamine, 25 mg./kg., was administered to two male dogs. The experiment lasted 8 weeks.

The autonomic effects were closely similar to those observed during the preliminary experiment. The central nervous signs have already been described. We observed less dependence of the autonomic effects on dose than we expected. The ocular effects in particular were readily seen after doses of 5 mg./kg. pempidine or 26539, though the majority of the animals most affected received 25 mg./kg. The three drugs did not differ greatly in severity of action on the eye or on mouth or tongue.

Tolerance to the autonomic though not to the central nervous effects developed. The effects worsened during the first week, remained approximately similar during the second week, and began to decline during the third week. This decline was obvious during the fourth week, but thereafter little further change occurred. All signs could still be seen at any time during the experiment, particularly 1½ to 3 hr. after the time of dosing. The effect that seemed to decline most was dryness of the mouth and tongue.

Two dogs died. One was dog 981. The other, a bitch receiving pempidine, 25 mg./kg., developed what we think was a sterile pneumonia, and died 20 hr. after accidental tracheal dosing.

The initial weight range of the young adults used was 9.5 to 11.5 kg., and the groups were approximately matched. The following weight changes

were observed at 3 and 8 weeks respectively: pempidine, 25 mg./kg., −6.8%, −7.8% ; pempidine, 5 mg./kg., −0.4%, +5.6% ; 26539, 25 mg./kg., −7.7%, −5.6% ; 26539, 5 mg./kg., +8.7%, +12.8% ; mecamylamine, 25 mg./kg., −16.4% (two dogs), −5.5% (one dog) ; controls, +4.6%, +10.8%.

All drugs in doses of 25 mg./kg. caused a moderately severe loss of weight. Compound 26539, at 5 mg./kg., appeared to have promoted growth, and a similar effect was observed in rats given very small doses of 26539. This response might be associated with the pituitary changes caused by 26539, but it requires further study.

Haematological studies were carried out at fortnightly intervals. We could discern no effect of any drug on red or white cell count, or on haemoglobin concentration. The concentrations of cholesterol in blood specimens obtained at the time of autopsy were normal.

No gross changes were seen at autopsy in the dogs which were killed after 4 or 8 weeks, and our colleague Dr. G. E. Paget reported that no histopathological changes were seen in tissues (listed in the Method) of dogs receiving mecamylamine (see also Stone *et al.*, 1956) or pempidine. Histopathological studies of brains, cords, and eyes are still incomplete, and the pituitary glands were damaged slightly at autopsy and their assessment was difficult.

Compound 26539, 25 mg./kg., arrested spermatogenesis at the late spermatid stage in the single male dog that received this dose. This finding has strengthened our opinion that 26539 is unsuitable for trial in man.

Discussion

Mecamylamine, pempidine, and 1-ethyl-2:2:6:6-tetramethylpiperidine (26539) are qualitatively very similar. All the actions studied, except one discussed below, were common to all three. They are highly potent non-quaternary blocking agents active on sympathetic and on parasympathetic ganglia ; this action was slow in onset but was protracted. They were well absorbed from the gastrointestinal tract. They displayed central nervous actions of which the most obvious and probably the most important sign was tremor. They had feeble actions at the neuromuscular junction ; they reduced the amplitude and rate of beat of the isolated heart and reduced the coronary flow ; they had local anaesthetic activity if penetration was facilitated by intimate contact with nerve fibres or nerve endings. They lacked more than a trifling ability to inhibit excitatory responses elicited *in vitro by* acetylcholine, adrenaline, histamine, or 5-hydroxytryptamine. This novel pattern of action suggests that they may have a novel type of action. We support the mode of action proposed by Bennett *et al.* (1957) for mecamylamine, but are doubtful of the validity of some of their evidence, which was of two kinds, relating on the one hand to ganglia, on the other to the neuromuscular junction. The potency of all three compounds at the latter site was little more than one hundredth of that at the former, and we prefer not to assume that the two actions are allied, though they may be. The ganglionic evidence adduced by Bennett *et al.* (1957) is of two kinds: first, the duration of action of mecamylamine ; second, the maintenance of a sustained, unfatigued, response to preganglionic stimulation during partial mecamylamine blockade. On occasions we have observed a poorly sustained contraction during partial blockade with mecamylamine, and have been inclined to attribute its occurrence to the condition of the cat rather than to a specific ganglionic event. Whether such an effect during blockade by a conventional quaternary compound could be explained on similar lines is doubtful and discussion will be restricted to the evidence relating to the time course.

We have been more impressed by the slow onset of action than by the duration of action. A quaternary or other compound with conventional competitive blocking actions but which was not readily excreted or degraded could have prolonged action. Perhaps chlorisondamine might be such a compound and, if so, the duration of action need not be necessarily related to the mode of action. On the other hand competitive interference with synaptic transmission is almost certainly extracellular (Paton and Zaimis, 1952) and an exceptionally slow onset of interference is unlikely though not impossible. We were struck by the similarity in time-course between the ganglionic-blocking action of pempidine (Fig. 3) and the likely time-course of entry of substances of this pK_a into cells. This view was reinforced by the properties of the closely related compound 1:1:2:2:6:6-hexamethylpiperidinium, the action of which was rapid in onset and brief in duration, and which we presumed acted extracellularly. The interesting hypothesis of Bennett *et al.* (1957), that the postganglionic cell is altered by the entry of mecamylamine so that its responses to acetylcholine, etc., are modified, consequently seems very attractive. There is, however, some evidence against it. Payne and Rowe (1947) have shown that carbon dioxide, administered after very large doses of mecamylamine, caused an increase in

the plasma concentration of mecamylamine, a brisk hypotension, and an increased neuromuscular block. The simplest explanation of these findings, put forward by Payne and Rowe (1957), is that mecamylamine is active in the extracellular phase, and that carbon dioxide, by lowering the plasma *p*H, causes its passage from the cells into the extracellular phase, where increased activity is then displayed. The theory of extracellular action on ganglia could also explain the large transient hypotensive responses which Payne and Rowe (1957) observed after each of the large serial doses of mecamylamine. However, Payne and Rowe (1957) suggested an alternative explanation which is not against the intracellular hypothesis, namely, that there is a direct vasodilator action of CO_2 during complete sympathetic blockade, the transient hypotensive responses to each new large dose of mecamylamine being attributed to myocardial depression. We did not see large transient hypotensive responses to pempidine given in large serial doses (Fig. 11). Since pempidine is so similar to mecamylamine in ganglionic actions we think, therefore, that the large hypotensive responses to successive doses of mecamylamine were not ganglionic in origin. The possibility that they were due to an effect on the heart receives some support from our evidence of the lower toxicity to the heart of pempidine. In our opinion, the balance of the present evidence, so far as hypotensive and ganglionic actions are concerned, favours an intracellular action of mecamylamine and pempidine.

Let us turn next to consider the comparative values of the three drugs. Our results in this connexion have been summarized in Table VII, in which comparative activities and toxicities of pempidine and 26539 are expressed as % of those of mecamylamine. Consider first pempidine and mecamylamine. Desirable ganglionic actions, type A, are those relating to the hypotensive action. By the intravenous route, the results of the four tests are in accord, making pempidine about twice as active as mecamylamine. Unwanted ganglionic actions, type B, are those likely to cause clinical side-effects such as constipation, paralytic ileus, tachycardia, urinary retention, etc. Most of our tests appeared to endow pempidine with some advantage over mecamylamine in that it was less than twice as active, but these particular tests involved absorption. Since other results, notably ratios of parenteral to oral toxicity, suggested that pempidine is somewhat less rapidly absorbed (or more rapidly eliminated) than mecamylamine, we think that we have obtained no conclusive evidence of superiority of the former in respect of

TABLE VII

ACTIVITY AND TOXICITY OF DRUGS EXPRESSED AS % EFFECT OF MECAMYLAMINE

Estimates given within brackets are approximate. Where two salts of pempidine and 26539 were examined, only values for hydrogen tartrates are given. Type A actions are those regarded as desirable ganglionic actions; Type B, unwanted ganglionic actions; Type C, unwanted non-ganglionic actions.

Type of Action	Preparation or Test Used	Activity or Toxicity	
		Pempidine	26539
A	Cat nictitating membrane	240	310
	Pressor response to phenyldimethylpiperazinium	230	135
	Pressor response to acetylcholine	(200)	(200)
	Carotid occlusion	(200)	(200)
B	Vagal bradycardia	(200)	—
	Phenyldimethylpiperazinium on guinea-pig ileum *in vitro*	250	180
	Gastric secretion, s.c.	100	260
	Gastric secretion, intraduodenal	130	260
	Faecal weights	110	200
	Urinary retention, mouse	30	—
C	Tremor, mouse	40	50
	Acute toxicity: LD50, i.v.	35	55
	,, ,, i.p.	40	85
	,, ,, oral	25	70
	Rat growth	20	45
	Local anaesthesia	85	65
	Cardiotoxicity *in vitro*	(40)	—

actions of type B. This view is supported by the results of the vagal bradycardia and guinea-pig ileum tests, both of which gave potency ratios of about two and neither involved absorption. A possible exception is that in the intact mouse, pempidine caused much less urinary retention than did mecamylamine. Nevertheless, it had as large an effect on the response of cat bladder to dimethylphenylpiperazinium as it had on the pressor response to this drug, and it is possible that the lower potency in the intact animal is due to a non-ganglionic action of one of the two drugs. We conclude that pempidine has no specificity for sympathetic as against parasympathetic ganglia, and that clinical side-effects caused by anti-parasympathetic actions are to be expected.

Unwanted non-ganglionic actions of type C show that pempidine has several important advantages over mecamylamine. For instance, the lethal dose was much higher; the dose causing tremor was higher; the dose depressing rat growth was much higher. If the % potencies in Table VII type C are compared with those derived from ganglionic actions under circumstances involving oral absorption, that is to say, actions on gastric secretion and on faecal weights, then pempidine has a safety margin about three times that of mecamylamine in respect of tremor, five times in respect of death, and six times in respect of general systemic toxicity as indicated by rat

growth. This applies equally to compound 26539. The results in Table VII type B suggest that this drug is probably more rapidly absorbed than pempidine and that the safety margins named above are about 5, 3½, and 5 times respectively. On the one hand, the quantitative findings considered in isolation therefore support clinical test of 26539 equally with that of pempidine. On the other hand, the qualitative histopathological observations of our colleague, Dr. Paget, plainly indicate that such tests on 26539 would be potentially hazardous. Pempidine and mecamylamine were remarkable in that they lacked toxic actions that are demonstrable histopathologically whereas 26539 caused changes in the pituitary gland and in the testis which entirely precluded clinical trial until these effects and their causes have been elucidated. This evidence has strengthened our opinion that clinical tests based on quantitative findings alone may be dangerous and should not be undertaken. The minimal precautions that we consider proper were summarized recently by Paget and Spinks (1958).

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THE NEUROMUSCULAR BLOCKING ACTION OF BENZOQUINONIUM CHLORIDE IN THE CAT AND IN THE HEN

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Benzoquinonium (Mytolon) has been shown to produce a curare-like rather than a decamethonium-like paralysis of neuromuscular transmission in the tibialis anterior and soleus muscle of cats and the gastrocnemius muscle of hens. In the cat, but not in the hen, benzoquinonium had an additional action in preventing both the twitch potentiating action and the anti-curare action of edrophonium, neostigmine, eserine, and tetraethyl pyrophosphate. Paralysis produced by benzoquinonium was antagonized by injected acetylcholine and by acetylcholine liberated by tetanic stimulation of the motor nerve but, in the cat, anticholinesterases were without effect even when they were first administered in circumstances in which acetylcholine accumulation might be expected to occur. These findings suggested that inhibition of cholinesterase played little part in the skeletal muscle effects of the anticholinesterases studied. Although anticholinesterases did not antagonize benzoquinonium paralysis in the cat, they nevertheless potentiated the antagonistic action of injected acetylcholine.

The muscle-paralysing action and other pharmacological properties of benzoquinonium, *p*-benzoquinone-2:5-bis-N-(3-aminopropyl)-benzyl-diethylammonium chloride, were originally investigated by Hoppe (1950, 1951) and the compound subsequently received clinical trial as a muscle relaxant in conjunction with surgical anaesthesia (Arrowood, 1951; Foldes, 1951; Dundee, Gray, and Rees, 1952; Hunter, 1952; Gordon, 1953).

The mechanism of its blocking action has not been fully established. In the frog, mouse, rabbit, dog (Hoppe, 1951), and man (Arrowood, 1951) it has been shown to produce effects similar to those of tubocurarine which interrupts neuromuscular transmission by raising the threshold of the motor end plates to acetylcholine. In the cat, however, its action is believed to resemble that of the depolarizing substance, decamethonium. This latter belief is based mainly on the observations of Randall (1951), who showed that, in the cat, paralysis produced by benzoquinonium, like that produced by decamethonium, is not antagonized but actually slightly increased by the administration of the anti-curare substance, edrophonium.

From the work of Zaimis and her colleagues (Buttle and Zaimis, 1949; Paton and Zaimis, 1952; Jewell and Zaimis, 1954; Zaimis, 1953, 1954, 1956–7), it is clear that if there is a depolarizing element present in the action of a neuromuscular blocking agent, it will be more evident in the tibialis anterior muscle of the cat and in certain avian muscles than in the muscles of most other species. The cat and the hen were therefore used in the present experiments in which the effects of benzoquinonium chloride were compared more fully with those of tubocurarine chloride and decamethonium iodide in an attempt to throw further light on its mode of action.

METHODS

Cats.—Cats were anaesthetized with chloralose (80 mg./kg.) injected into the subcutaneous vein of the fore-limb or into the internal saphenous vein. A hind limb was set up in a horizontal position on a Brown-Schuster myograph stand. Shielded silver electrodes were placed on the sciatic nerve and the nerve was ligated central to the electrodes. Twitches and tetani of the tibialis anterior and soleus muscles were excited by rectangular pulses of 0.2 msec. duration and of twice the strength required to evoke a maximal twitch. The muscles were attached to flat steel springs and their contractions recorded on a smoked paper. For experiments on denervated muscles, the sciatic nerve on one side was divided

high in the thigh under sodium pentobarbitone anaesthesia. Degeneration was allowed to proceed for from 14 to 21 days after which the animal was anaesthetized with chloralose as described above. Direct stimuli of maximal strength and of 0.5 msec. duration were applied between the tendons of the muscles and the drill in the femur. Electrical contact with the tendons was made through shielded silver wires attached to saline-soaked pads of cotton wool. In order to ensure that the current supplied for direct stimulation was not short-circuited through the other tissues, the muscles were insulated by enclosing their bellies in rubber finger-stalls smeared with liquid paraffin. Drugs were injected intravenously through a cannula in the jugular vein or intra-arterially through a cannula in the cut central end of a branch of the femoral artery. When close-arterial injections to the tibialis anterior muscle were made, the method described by Brown (1938) was used.

Hens.—Hens were anaesthetized by the slow injection of pentobarbitone into a wing vein. When a sufficient depth of anaesthesia was reached (usually after the injection of about 40 mg./kg.) a similar quantity of pentobarbitone was injected intramuscularly. Twitches and tetani of the gastrocnemius muscle were elicited indirectly in the same way as in the experiments on cats. Drugs were injected intravenously through a cannula in the jugular vein.

Solutions of all drugs were made up in 0.9% w/v NaCl solution.

Results

After the administration of several doses of benzoquinonium, a gradual fall in blood pressure occurred and death occasionally ensued due to circulatory collapse. These effects were prevented by previous atropinization, and for this reason atropine (1 to 2 mg./kg.) was administered intravenously at the beginning of many of the experiments. Such treatment did not, however, alter the results to be described. Similar circulatory effects were described in man by Dundee *et al.* (1952).

In the cat, benzoquinonium chloride administered intra-arterially in doses of 30 to 50 μg. or intravenously in doses of 0.25 to 0.3 mg./kg. caused a 90 to 100% paralysis of the indirectly excited maximal twitches of the tibialis anterior and soleus muscles. In all the experiments benzoquinonium was approximately twice as potent by weight as tubocurarine. Like tubocurarine (Paton and Zaimis, 1951), benzoquinonium paralysed the soleus to a slightly greater extent than the tibialis anterior muscle (Fig. 7*a*) and paralysis of the respiratory muscles accompanied the effect on the limb muscles. In contrast, decamethonium had little effect on the soleus and respiratory muscles in doses which produced a 90 to 100% paralysis of the tibialis anterior (see also Paton and Zaimis, 1951). Potentiation of the maximal twitch and muscle fasciculations, such as are observed after injection of decamethonium (Paton and Zaimis, 1949), never followed the administration of benzoquinonium. During complete block of the indirectly excited maximal twitches, the muscles

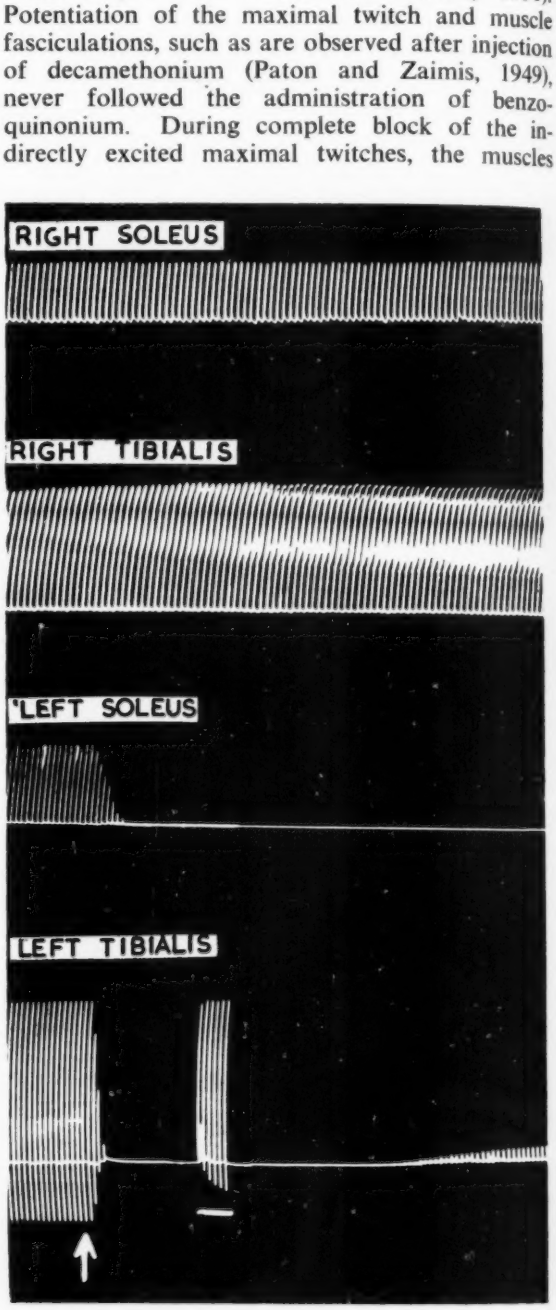

FIG. 1.—Cat 2.8 kg. Simultaneous recording of maximal twitches of both tibialis anterior muscles and both soleus muscles once every 10 sec. The muscles of the right leg were chronically denervated (18 days) and directly stimulated. Those of the left leg were normal and stimulated indirectly. At the arrow, 1 mg. of benzoquinonium was administered intravenously. During period marked by horizontal bar, left tibialis anterior was stimulated directly.

were still able to respond normally to direct stimulation (Fig. 1) and the contractions of the directly stimulated chronically denervated muscles were unaffected by benzoquinonium (Fig. 1) showing that the paralysis is not brought about through an action on the muscle fibre itself. Unlike decamethonium and other depolarizing substances, benzoquinonium even in large doses of up to 1 mg./kg. intravenously did not cause contracture in chronically denervated muscles (Fig. 1).

Benzoquinonium, tubocurarine, and decamethonium blocked the response to close-arterial injection of acetylcholine to a greater extent than the responses to maximal indirect stimuli. This effect with benzoquinonium is illustrated by Fig. 2 and shows that a reduction in the quantity of acetylcholine liberated from nerve terminals cannot play a major part in its blocking action.

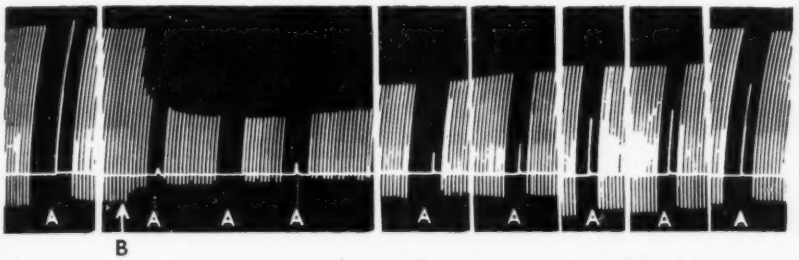

FIG. 2.—Cat 3.4 kg. Maximal twitches of the tibialis anterior muscle were elicited indirectly once every 10 sec. At *A*, electrical stimulation was stopped and 5 μg. of acetylcholine was injected close-arterially. At *B*, 20 μg. of benzoquinonium was injected close-arterially.

In the hen, the intravenous administration of benzoquinonium in doses of 150 to 200 μg./kg. caused a 90 to 100% paralysis of the indirectly excited maximal twitches of the gastrocnemius muscle. A similar degree of paralysis but of longer duration was produced by the intravenous administration of 0.8 to 1 mg./kg. of tubocurarine. The administration of benzoquinonium or tubocurarine reduced or abolished the contracture produced by a subsequent injection of decamethonium but did not alter the character of the response (Fig. 3) as does the previous administration of mecamylamine (Bennett, Tyler, and Zaimis, 1957). These results taken together demonstrate the absence of a depolarizing action of benzoquinonium and indicate that it produces a curare-like rather than a decamethonium-like paralysis of neuromuscular transmission.

In both the cat and the hen, cross cumulative effects occurred between benzoquinonium and tubocurarine when doses of each were administered alternately and, when either substance was administered at the peak of the effect of the other, an increase in the degree of block occurred. The additive effects of benzoquinonium and tubocurarine in the cat are illustrated in Fig 4*a*. On the other hand, benzoquinonium and decamethonium were seen to be mutually and markedly antagonistic (Fig. 4*b*).

Like the effect of tubocurarine and in contrast to that of decamethonium, neuromuscular block produced by benzoquinonium was antagonized by the administration of suxamethonium (20 to 30 μg./kg. intravenously), acetylcholine (30 μg. intra-arterially) or KCl (15 mg. intra-arterially). Paton and Zaimis (1949) used the response to tetanic stimulation of the motor nerve as an indication of the mode of action of neuromuscular blocking substances and the results of such a test are illustrated by Fig. 5. During partial paralysis by decamethonium, tetanic tension was fairly well maintained and after the tetanus there was no antagonism of the block. During partial paralysis by tubocurarine or benzoquinonium, however, tetanic stimulation produced only a brief "twitch-like" response from the muscle and after

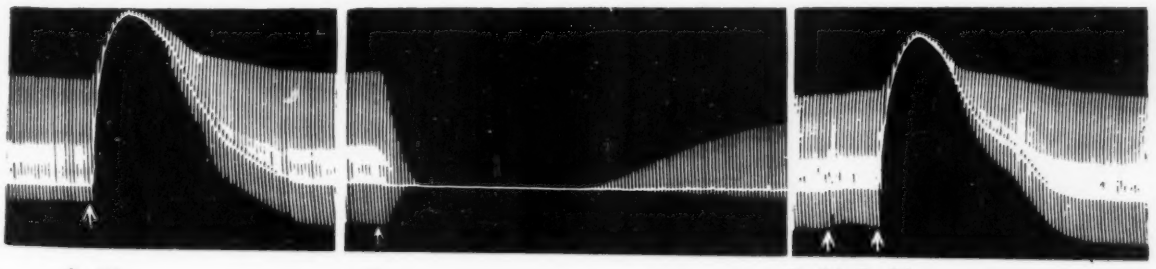

FIG. 3.—Hen 2.7 kg. Maximal twitches of the gastrocnemius muscle were elicited indirectly once every 10 sec. At $C_{10}(1)$, 40 μg. of decamethonium; at $C_{10}(2)$, 100 μg. of decamethonium; and at *B*, 0.4 mg. of benzoquinonium were injected intravenously.

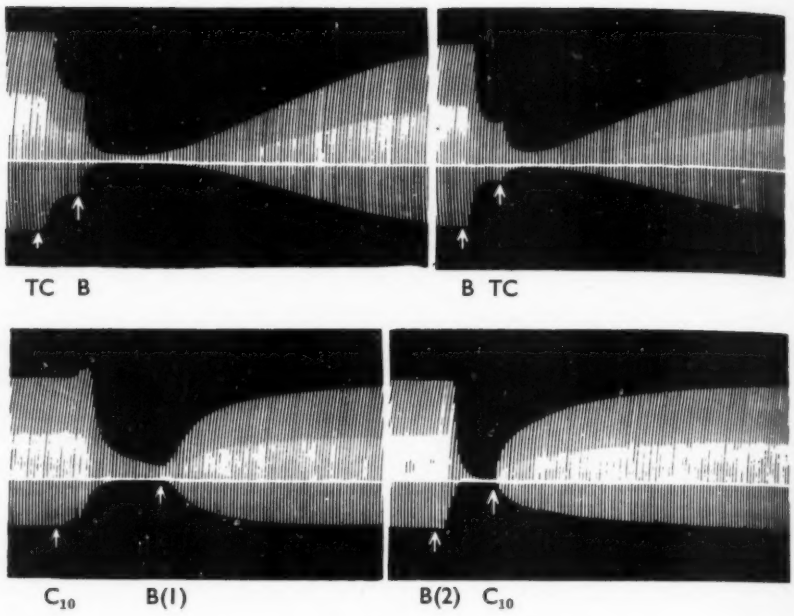

FIG. 4.—*a*, cat 3.8 kg. *b*, cat 2.9 kg. Maximal twitches of the tibialis anterior muscles were elicited indirectly once every 10 sec. At ***TC***, 0.4 mg. of tubocurarine. At ***B***, 0.25 mg., at ***B(1)***, 0.2 mg., and at ***B(2)***, 0.6 mg. of benzoquinonium. At C_{10}, 100 μg. of decamethonium. All injections were made intravenously.

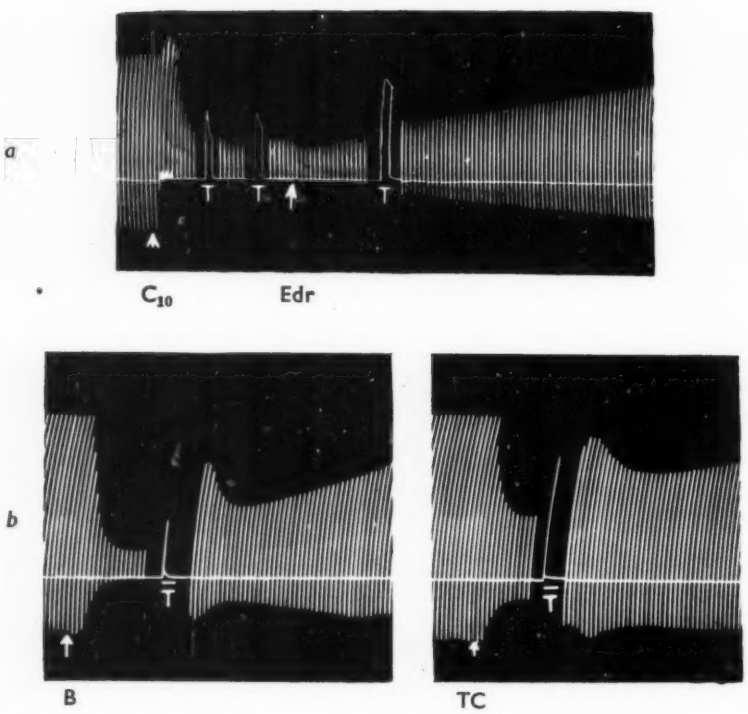

FIG. 5.—*a*, cat 2.7 kg. *b*, cat 3.4 kg. Maximal twitches of the tibialis anterior muscles were elicited indirectly once every 10 sec. At C_{10}, 100 μg. of decamethonium; at ***Edr***, 0.3 mg. of edrophonium; at ***B***, 0.6 mg. of benzoquinonium; at ***TC***, 0.8 mg. of tubocurarine. All injections were made intravenously. ***T*** indicates tetanus of 30 sec. duration and at a frequency of 50/sec.

the tetanus the block was markedly antagonized.

It has long been known that adrenaline antagonizes a partial paralysis produced by tubocurarine when it is administered during the effect (Rosenblueth, Lindsley, and Morison, 1936). No such antagonism occurs, however, when decamethonium is used (West and Zaimis, 1949). On the other hand, when adrenaline is administered first, the blocking action of tubocurarine is potentiated, while that of decamethonium is reduced (Paton and Zaimis, 1950). These results were confirmed in the present experiments on the cat and it was shown that in this respect, also, benzoquinonium resembled tubocurarine (Fig. 6). As with tubocurarine, the antagonistic action of adrenaline to benzoquinonium paralysis was more marked at higher rates of stimulation.

Bennett *et al.* (1957) have shown that the previous administration of mecamylamine potentiates the blocking action of tubocurarine but reduces that of decamethonium, at the same

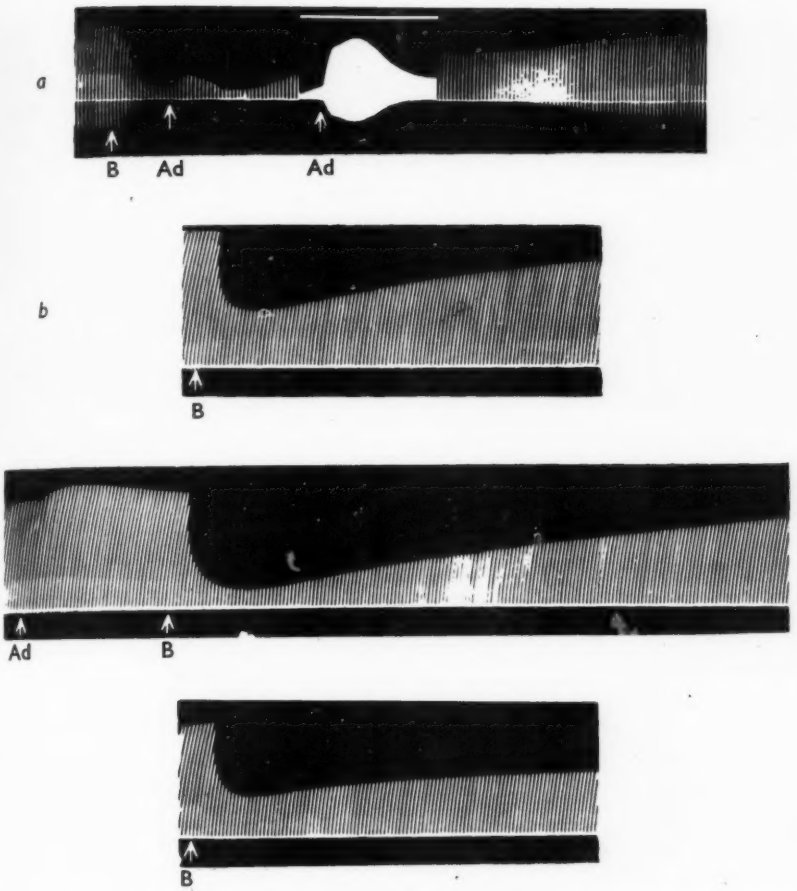

FIG. 6.—*a*, cat 2.4 kg. *b*, cat 3.1 kg. Maximal twitches of the tibialis anterior muscles were elicited indirectly once every 10 sec. At ***B***, 0.5 mg. of benzoquinonium, and at ***Ad***, 50 μg. of adrenaline, were administered intravenously. In *a* during period marked by horizontal bar the frequency of stimulation was increased to 60/min. In *b*, 90 min. elapsed between injections of benzoquinonium.

time altering the character of the response. These results were confirmed and again benzoquinonium was shown to resemble tubocurarine, its effect being potentiated by mecamylamine.

The results so far described show that benzoquinonium produced a curare-like paralysis of neuromuscular transmission, but in spite of this, as Randall (1951) also demonstrated, its effect in the cat is not antagonized by edrophonium.

In addition to edrophonium, neostigmine, eserine and tetraethyl pyrophosphate were studied. In some experiments edrophonium (30 to 300 μg./kg.), neostigmine (30 to 300 μg./kg.), eserine (50 to 300 μg./kg.) or tetraethyl pyrophosphate (0.5 to 1 mg./kg.) was administered intravenously at the peak of the paralysis produced either by tubocurarine or by benzoquinonium. In others a small dose of either benzoquinonium or tubocurarine was administered at a constant time interval, which varied in different experiments from 30 min. to 4 hr., and edrophonium, neostigmine, eserine or tetraethyl pyrophosphate was injected 1 to 20 min. before the third or fourth dose of the blocking agent. All four substances readily antagonized tubocurarine in both types of experiment, but in the cat no antagonism to benzoquinonium could be demonstrated by either method (Fig. 7). In fact, when administered during the paralysis, edrophonium and neostigmine often slightly increased the block produced by benzoquinonium.

During these experiments it was observed that the administration of edrophonium, neostigmine, or eserine during partial block produced by tubocurarine was without effect in cats which had previously received benzoquinonium. Fig. 7*a* and *b* illustrates this effect in the case of edrophonium and neostigmine. This abolition of the anticurare effect lasted approximately 2 hr. after the intravenous administration of 0.2 mg./kg. of benzoquinonium. After the administration of benzoquinonium, edrophonium and neostigmine usually slightly deepened the paralysis produced by tubocurarine. Large doses of tetraethyl pyrophosphate (1 mg./kg.) still produced some antagonism, however, although the effect was reduced (Fig. 7*e*). Owing to the long duration of action of tetraethyl pyrophosphate, its anti-curare action showed marked tachyphylaxis. Accurate conclusions could not, therefore, be drawn from experiments in which its anti-curare action, both before and after the administration of benzoquinonium, was studied in the same animal. However, in an animal which had previously received benzoquinonium, the anticurare action of the first injection of tetraethyl pyrophosphate was found to be non-existent or weak compared with control responses studied in different animals (Fig. 7*c* and *e*).

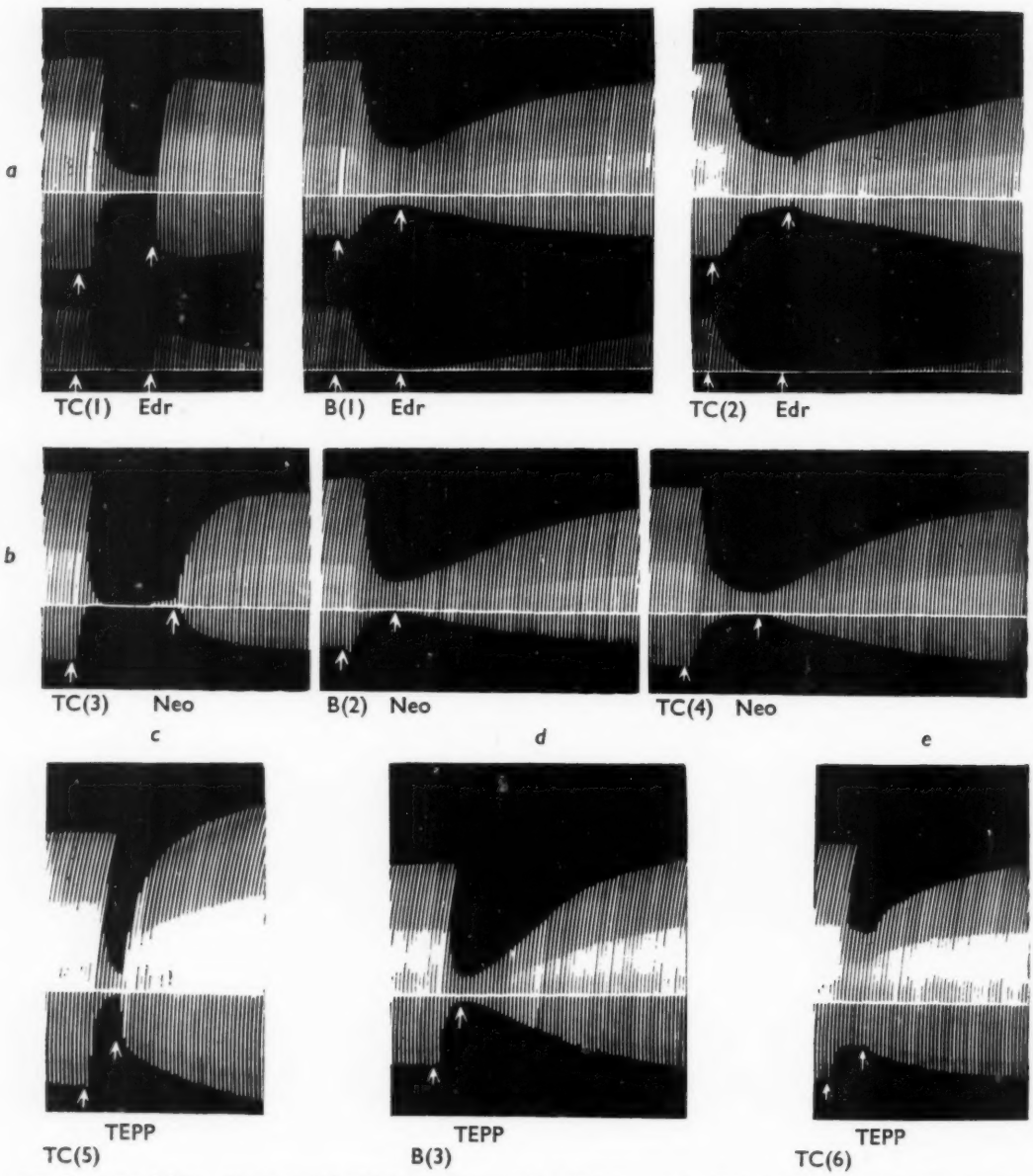

FIG. 7.—*a*, cat 3.0 kg. Upper record, tibialis anterior muscle, and lower record, soleus muscle. *b*, cat 3.4 kg. tibialis anterior muscle. *c*, cat 3.7 kg. tibialis anterior muscle. *d*, cat 2.9 kg. tibialis anterior muscle. *e*, cat 3.2 kg. tibialis anterior muscle. All records are of maximal twitches elicited indirectly once every 10 sec. At ***TC(1)*** 1 mg., at ***TC(2)*** 0.6 mg., at ***TC(3)*** 1.2 mg., at ***TC(4)*** 0.9 mg., at ***TC(5)*** 1.3 mg. and at ***TC(6)*** 0.8 mg. of tubocurarine respectively. At ***Edr***, 0.3 mg. of edrophonium; at ***Neo***, 0.15 mg. of neostigmine; at ***TEPP***, 3 mg. of tetraethyl pyrophosphate. At ***B(1)*** 0.3 mg., at ***B(2)*** 0.5 mg. and at ***B(3)*** 0.6 mg. of benzoquinonium. All injections were given intravenously. In *a* and *b*, 1 hr. elapsed between the doses of the blocking agents. In *e*, 0.5 mg. of benzoquinonium had been administered 45 min. previously.

This prevention of the anti-curare action was studied in another way, namely sufficient tubocurarine to produce a 50 to 70% paralysis of the maximum twitches was injected and, at the peak of the effect, a small dose of benzoquinonium was administered, which was insufficient by itself to produce any decrease in the maximal twitch tension. This was followed by the administration of either edrophonium, neostigmine, eserine, or tetraethyl pyrophosphate, none of which showed any antagonistic action in spite of the fact that under these circumstances the majority of the

muscle fibres must have been blocked by tubocurarine. Although after the administration of benzoquinonium, the anticholinesterases no longer antagonized tubocurarine, other antagonistic substances were still effective. Thus the paralysis was still antagonized by tetanic stimulation of the motor nerve and by the administration of acetylcholine, suxamethonium, decamethonium, KCl, and adrenaline. The lack of antagonism by anticholinesterases is not, therefore, the result of a change in the mechanism of tubocurarine blockade.

Small doses of benzoquinonium also prevented the potentiation of the unblocked maximal twitch produced by the anticholinesterases although with large doses of tetraethyl pyrophosphate some effect was still to be seen. Fig. 8 illustrates this effect with edrophonium.

It was considered possible that benzoquinonium in some way prevents the anticholinesterases from reaching their site of action in the cat. Experiments were therefore designed in which the anticholinesterases were administered to animals which had not previously received benzoquinonium. Under these circumstances acetylcholine accumulation should occur and some antagonism to subsequently administered benzoquinonium might be expected. These experiments were carried out as follows. In order to determine the sensitivity of the preparation to competitive blockade, 0.4 mg./kg. of tubocurarine

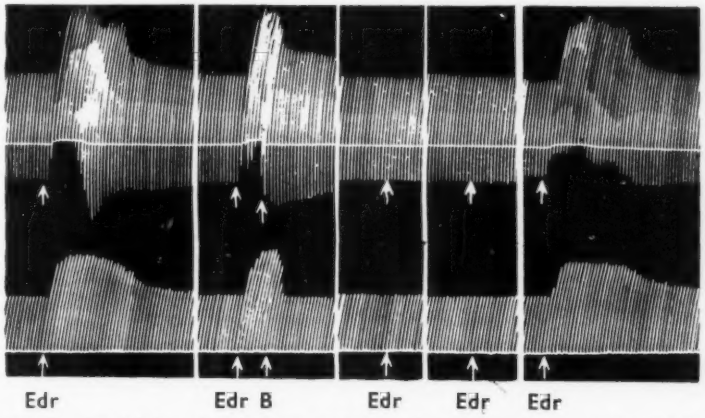

FIG. 8.—Cat 2.7 kg. Simultaneous recording of maximal twitches of the tibialis anterior muscle (upper record) and of the soleus muscle (lower record) elicited indirectly once every 10 sec. At ***Edr***, 0.3 mg. of edrophonium, and at ***B***, 0.15 mg. of benzoquinonium, were administered intravenously. 30 min. elapsed between each injection of edrophonium.

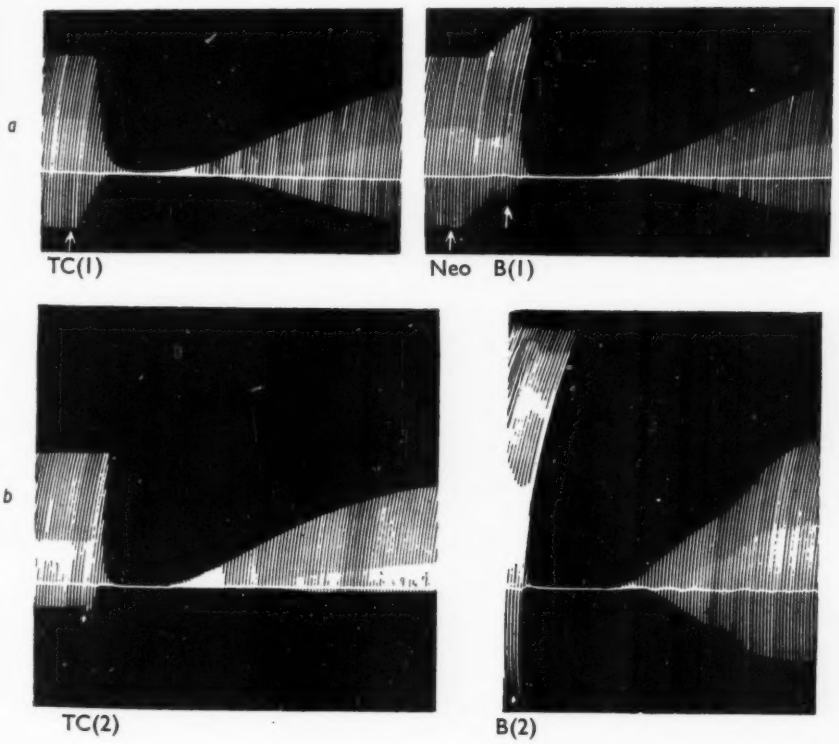

FIG. 9.—*a*, cat 3.4 kg. *b*, cat 3.2 kg. Maximal twitches of the tibialis anterior muscles were elicited indirectly once every 10 sec. At ***TC(1)*** 1.4 mg. and at ***TC(2)*** 1.3 mg. of tubocurarine. At ***B(1)*** 0.7 mg. and at ***B(2)*** 0.65 mg. of benzoquinonium. At ***Neo*** 0.2 mg. of neostigmine. 3 mg. of tetraethyl pyrophosphate was administered 15 min. before ***B(2)***. All injections were given intravenously. In both *a* and *b*, 4 hr. elapsed between the injections of the blocking agents.

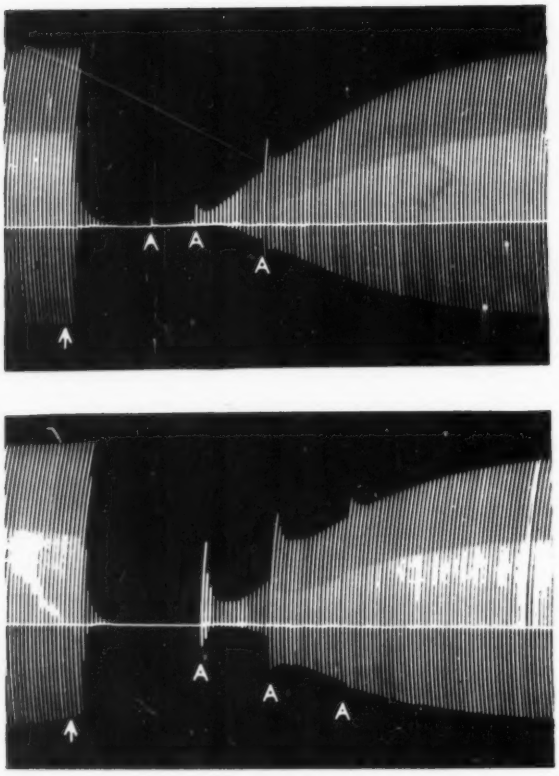

FIG. 10.—Cat 4.1 kg. Maximal twitches of the tibialis anterior muscle were elicited indirectly once every 10 sec. At the arrows, 0.7 mg. of benzoquinonium was administered intravenously. At *A*, 30 μg. of acetylcholine was administered intra-arterially. 10 min. before the second injection of benzoquinonium, 0.4 mg. of eserine was administered intravenously. 90 min. elapsed between the injections of benzoquinonium.

was administered intravenously. Four to five hours later neostigmine (100 μg./kg.), eserine (150 μg./kg.) or tetraethyl pyrophosphate (1 mg./kg.) was administered and this was followed 10 to 20 min. later by 0.2 mg./kg. of benzoquinonium. Since in all experiments benzoquinonium was twice as potent as tubocurarine, this dose would be expected to produce a comparable response. A long interval was allowed to elapse between the doses of the blocking agents in order to reduce cumulative effects to a minimum. Even under these circumstances, no antagonism to benzoquinonium could be demonstrated; in every case the paralysis produced was equal to, or slightly greater than, that produced by the initial injection of tubocurarine. Fig. 9 illustrates typical results with tetraethyl pyrophosphate and neostigmine.

Although in the cat edrophonium, neostigmine, eserine, and tetraethyl pyrophosphate did not antagonize paralysis produced by benzoquinonium, they nevertheless potentiated the antagonistic action of injected acetylcholine. Eserine and tetraethyl pyrophosphate were the most potent in this respect while the effect of edrophonium was weak or non-existent. The upper record of Fig. 10 illustrates the antagonistic action of injected acetylcholine against paralysis by benzoquinonium. Ten minutes before the second injection of benzoquinonium, eserine was administered, but in spite of this the paralysis was not reduced. The antagonistic action of injected acetylcholine, however, was markedly potentiated.

An interesting species difference was demonstrated by experiments on the hen in which edrophonium, neostigmine, eserine, and tetraethyl pyrophosphate readily antagonized paralysis produced both by tubocurarine and by benzoquinonium (Fig. 11).

DISCUSSION

The present experiments demonstrate that in the cat and the hen, as in other species, benzoquinonium caused a reversible paralysis of the neuromuscular junction typical in many respects of that produced by substances such as tubocurarine. That benzoquinonium did not possess a depolarizing action is shown by the lack of any muscle fasciculations or potentiation of the maximal twitch and by the absence of a contracture in avian or chronically denervated mammalian muscle. In fact, benzoquinonium and depolarizing substances such as decamethonium, suxamethonium, and acetylcholine were shown to be mutually antagonistic.

The administration of benzoquinonium, even in doses too small to affect the maximal twitch tension, was shown to reduce or abolish both the potentiating action on the maximal twitch and the anti-curare action of edrophonium, neostigmine, eserine, and tetraethyl pyrophosphate. These results show that, in addition to its neuromuscular blocking action, benzoquinonium prevents the skeletal muscle effects of these anticholinesterases in the cat. The fact that anticholinesterases did not antagonize paralysis produced in the cat by benzoquinonium appears, therefore, not to be a consequence of the mechanism of its blocking action as Randall (1951) believed and as is the case with decamethonium (Paton and Zaimis, 1949), but rather to be the result of this additional action.

Randall (1952) showed that the replacement of the benzyl groups of benzoquinonium with nitrobenzyl or cyano-benzyl radicals resulted in compounds, the blocking actions of which were antagonized by edrophonium in the cat. Ran-

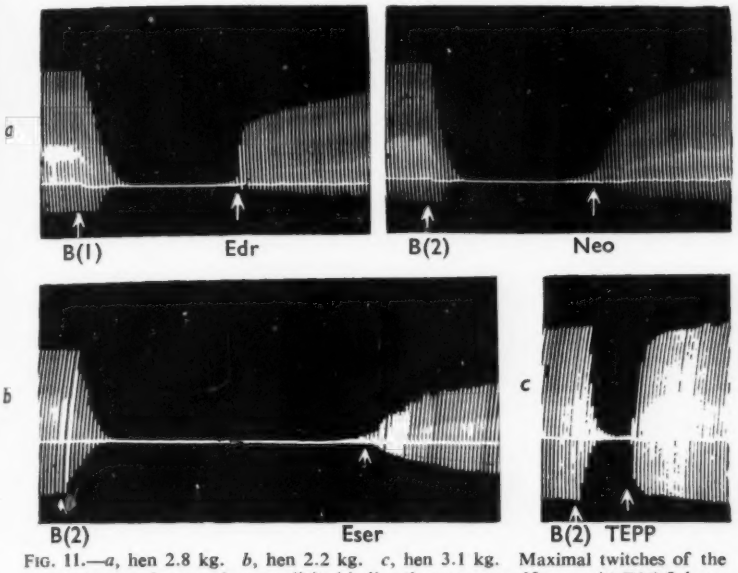

FIG. 11.—*a*, hen 2.8 kg. *b*, hen 2.2 kg. *c*, hen 3.1 kg. Maximal twitches of the gastrocnemius muscle were elicited indirectly once every 10 sec. At ***B(1)*** 0.4 mg. and at ***B(2)*** 0.5 mg. of benzoquinonium. At ***Edr*** 0.3 mg. of edrophonium. At ***Neo*** 0.15 mg. of neostigmine. At ***Eser*** 0.4 mg. of eserine. At ***TEPP*** 3 mg. of tetraethyl pyrophosphate. All injections were made intravenously.

dall interpreted this as a conversion from a decamethonium-like to a curare-like substance, but the present experiments indicate that such substitution probably abolished the "anti-edrophonium" effect without altering the mechanism of the blocking action. Hoppe, Funnell, and Lape (1953, 1955) showed that the replacement of the benzyl groups with ethyl groups also resulted in a compound which was antagonized by edrophonium so that it appears that the presence of unsubstituted benzyl groups on the quaternary nitrogen centres of benzoquinonium is an important factor in the "anti-anticholinesterase" effect in cat skeletal muscle.

Although benzoquinonium has been shown to produce a curare-like paralysis in all the species studied, considerable species difference exists with regard to the effects of anticholinesterases. The present experiments have shown that while edrophonium, neostigmine, eserine, and tetraethylpyrophosphate are completely ineffective in the cat, they are all effective antagonists to benzoquinonium in the hen. Other species appear to lie between these extremes; for example, it has been reported that in the dog (Hoppe, 1951) and the rabbit (Hoppe *et al.*, 1955) neostigmine was slightly effective particularly when it was administered before benzoquinonium. Edrophonium, however, was without effect in the rabbit. According to Dundee *et al.* (1952), neostigmine antagonized paralysis produced by benzoquinonium in man.

Hoppe (1951) has shown from *in vitro* studies that benzoquinonium itself possesses some anticholinesterase activity. A potent inhibitor of the acetylcholinesterase at the neuromuscular junction might, by inhibiting all the enzyme in the region, prevent the effects of other anticholinesterases administered subsequently. The present experiments showed, however, that even when edrophonium, neostigmine, eserine, or tetraethyl pyrophosphate was administered first, the paralysing action of benzoquinonium was not reduced. Furthermore, in some recent experiments it has been shown that the previous administration of the potent anticholinesterases, eserine and neostigmine, does not abolish the antagonism to tubocurarine produced by edrophonium or tetraethyl pyrophosphate (Bowman, unpublished observations). These results therefore supply evidence that the anticholinesterase action of benzoquinonium cannot be the explanation for the failure of edrophonium, neostigmine, eserine, and tetraethyl pyrophosphate to antagonize its blocking action.

From a chemical point of view, it is unlikely that benzoquinonium can reactivate an inhibited enzyme or that it can combine chemically in the blood with all of the different types of anticholinesterase studied; the fact that the anticholinesterases are active antagonists to benzoquinonium in the hen is also evidence against such a possibility.

Neuromuscular block produced by benzoquinonium is antagonized by injected acetylcholine and by acetylcholine liberated from the motor nerve during tetanus, and yet, in the cat, anticholinesterases are without effect even when they are administered first. These facts suggest that inhibition of cholinesterase and consequent acetylcholine accumulation is not the main mechanism responsible for the skeletal muscle effects of edrophonium, neostigmine, eserine, and tetraethyl pyrophosphate. Some workers (Riker, Wescoe, and Brothers, 1949; Wescoe and Riker, 1951; Riker, 1953) have expressed the opinion that the main action of edrophonium, neostigmine and similar compounds in skeletal muscle is a direct acetylcholine-like stimulation of the motor end plate. The work of others (Randall and Lehmann,

1950; Randall, 1950; Hobbiger, 1952) does not support this conclusion and the present experiments also rule out such a possibility since substances which are known to possess such an action (decamethonium, suxamethonium, and acetylcholine itself) readily antagonize benzoquinonium paralysis while edrophonium and neostigmine do not. Riker, Roberts, Standaert, and Fujimori (1957) have recently put forward evidence that the repetitive firing seen in the motor nerves of cats under the influence of compounds of the edrophonium and neostigmine type is not, as is generally believed, entirely the result of retrograde activation of nerve by muscle but rather that it is partly brought about by an action of such substances on the motor nerve terminal. They concluded that, as a consequence of this action, neuromuscular transmission is facilitated and they summarized some of the evidence from other sources which points to a similar action of eserine and dyflos. Douglas and Paton (1954), in their study of the motor end plate depolarization produced by tetraethyl pyrophosphate in the cat, pointed out that they had no evidence to exclude the possibility that tetraethyl pyrophosphate causes a release of acetylcholine from nerve. Such an action would account for the skeletal muscle effects of anticholinesterases and would imply that whatever change occurs, when the nerve impulse reaches the nerve ending, is exaggerated or prolonged so that under the influence of these substances a greater quantity of acetylcholine is released by each stimulus. If such is the case, benzoquinonium, in addition to its action at the motor end plate, might inhibit the skeletal muscle effects of anticholinesterases by competing with them for receptor sites on the motor nerve terminal.

The anticholinesterase action of benzoquinonium which was demonstrated by Hoppe (1951), and which is presumably responsible for the muscarinic effects observed in man (Dundee *et al.*, 1952) and in the cat, may therefore merely be a reflexion of the other action. It is not unlikely that a substance which prevents the action of a second substance at one site may have a similar action to the second substance at another. The present experiments showed that neostigmine, eserine, and tetraethyl pyrophosphate in the presence of benzoquinonium still potentiate the skeletal muscle effects of injected acetylcholine, and experiments now in progress indicate that benzoquinonium does not prevent the muscarinic effects of anticholinesterases. The conclusion is therefore drawn that benzoquinonium produces a curare-like blockade of the neuromuscular junction and in addition, in the cat, it prevents the skeletal muscle effects of edrophonium, neostigmine, eserine, and tetraethyl pyrophosphate without abolishing their anticholinesterase activity.

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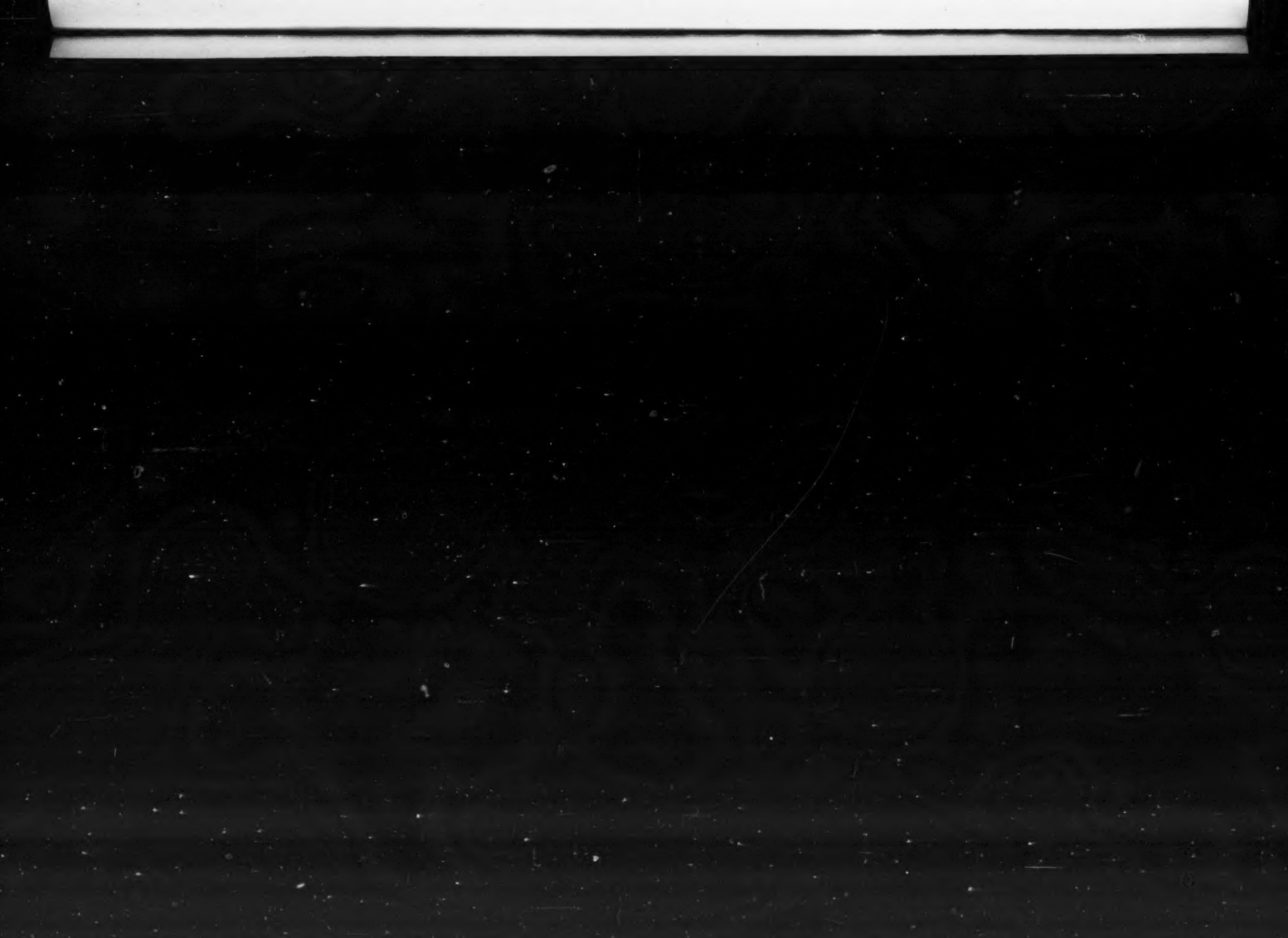

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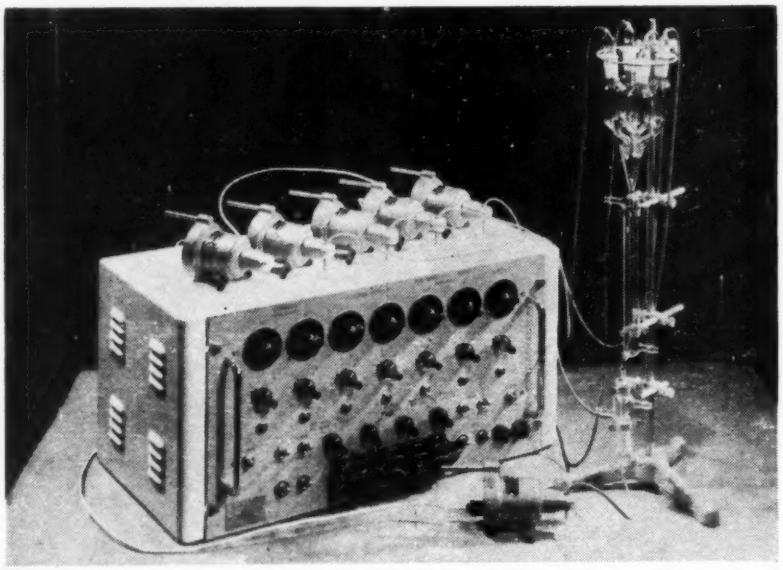

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